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(54) Title: PRIMERS FOR THE PCR AMPLIFICATION OF METASTATIC SEQUENCES

(57) Abstract

The invention relates to methods for the detection of metastatic diseases such as metastatic prostate, breast and lung carcinoma, in fresh or fixed biological samples. Nucleic acids are purified from fixed samples of patient tissue or fluid suspected to contain metastatic tissue and reverse transcribed to cDNA. Metastatic-specific sequences within the cDNA are amplified by polymerase chain reaction. Primers for PCR amplification comprise sequences from the exonic regions of a metastatic-specific expression product that span regions of expressed RNA that are not degraded even after long-term storage in paraffin. Metastatic-specific expression products include, for example, nucleic acids that contain sequences of the prostate specific antigen gene for use in the detection of metastatic prostate carcinoma. The resulting amplified nucleic acid sequences will include metastatic-specific sequences which can be easily detected. The invention also relates to nucleic acid primers which can be used for PCR amplification of metastatic-specific sequences such as sequences which correspond to the exonic regions of the PSA gene, and to kits which comprise these primers and other reagents useful for the rapid detection of metastatic diseases.

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PRIMERS FOR THE PCR AMPLIFICATION OF METASTATIC SEQUENCES

Background

1. Field of the Invention

This invention relates to methods for the sequence-specific amplification of metastatic nucleic acids in the detection and screening of metastatic diseases from biological samples such as metastatic neoplasms of the prostate. The invention also relates to nucleic acid primers specific to tissue-specific antigens such as prostate specific antigen and to kits containing these primers. Such kits can be used to amplify prostate specific antigen nucleic acids and provide an effective means to detect heretofore undetectable metastatic prostatic neoplasia.

2. Description of the Background

The walnut-sized prostate is an encapsulated organ of the mammalian male urogenital system. Located at the base of the bladder, the prostate is partitioned into zones referred to as the central, peripheral and transitional zones, all of which surround the urethra. Histologically, the prostate is a highly microvascularized gland comprising fairly large glandular spaces lined with epithelium which, along with the seminal vesicles, supply the majority of fluid to the male ejaculate.

As an endocrine-dependent organ, the prostate responds to both the major male hormone, testosterone, and the major female hormones, estrogen and progesterone. Testicular androgen is considered important for prostate growth and development because, in both humans and other animals, castration leads to prostate atrophy and an absence of any incidence of prostatic carcinoma.

The major neoplastic disorders of the prostate are benign enlargement of the prostate, also called benign prostatic hyperplasia (BPH), and prostatic carcinoma. BPH is very common in men over the age of 50. It is characterized by the presence of a number of large distinct nodules in the periurethral area of the prostate. Although benign, these nodules can produce obstruction of the urethra causing nocturia, hesitancy to void, and difficulty in obstruction of the urethra causing nocturia, hesitancy to void, and difficulty in starting and stopping a urine stream upon voiding the bladder. These conditions could also exist in prostatitis which implies an associated inflammation possibly due to infection. Occasionally, catheterization is required and even surgery. In the more extreme cases, secondary changes in the bladder can occur such as hypertrophy, acute retention with secondary upper urinary tract involvement, azotemia and uremia. Although all of these changes of the prostate may suggest pre-malignancy, there is as yet no direct association between nodular hyperplasia and prostatic carcinoma.

Carcinoma of the prostate is the most common form of cancer in human males. In the United States there are approximately 130,000 cases of prostate cancer yearly of which approximately 30,000 will be terminal (G.L. Lu-Yao et al., J.A.M.A. 269:2633-36, 1993). Histologically, most lesions are adenocarcinomas with well-defined gland patterns, but the more typical malignancy patterns associated with the very aggressive cancers are also common. Except in rare instances, all forms of prostatic carcinoma originate in the peripheral zone of the gland which is palpable upon rectal examination.

Prostatic carcinomas are staged by number and letter according to histological criteria such as the arrangement and appearance of malignant glands, and the degree of anaplasia of the cancerous cells. Stage A tumors include the incidental or clinically unsuspected cancers. These are detected in autopsy or, more commonly, after trans-urethral resection of the prostate for benign prostatic hyperplasia and rarely pose a problem to the patient. Stage B tumors are detectable by rectal digital examination and are also confined to the prostate. Tumors classified as B1, B2, and so on, indicate increasing volume of tumor formation. These tumors are fairly common in older men who begin to show signs and symptoms characteristic of some form of prostatic carcinoma. Stage C tumors have breached the prostate capsule and may or may not have invaded the surrounding tissues such as the seminal vesicles. Those tumors which have seminal vesicle involvement show an 80% correlation with lymph node involvement (C2). Stage D tumors have distinct metastases and a 100% correlation with lymph node involvement. Over 75% of patients with prostatic carcinoma show signs of stage C or D type development with significant urinary tract involvement. Only 5-10% of stage A patients, of those who have been followed for 8-10 years, develop stage C or D type prostatic carcinoma although the probability increases for patients who first present at a fairly young age.

In its more aggressive form, transformed prostatic tissue escapes from the prostate capsule and metastasize invading locally and throughout the bloodstream and lymphatic system. Metastasis, defined as tumor implants which are discontinuous with the primary tumor, can occur through direct seeding, lymphatic spread and hematogenous spread. All three routes have been found to occur with prostatic carcinoma. Local invasions typically involve the seminal vesicles, the base of the urinary bladder, and the urethra. Direct seeding occurs when a malignant neoplasm penetrates a natural open field such as the peritoneal, pleural or pericardial cavities. Cells seed along the surfaces of various organs and tissues within the cavity or can simply fill the cavity spaces. Hematogenous spread is typical of sarcomas and carcinomas. Hematogenous spread of prostatic carcinoma occurs primarily to the bones, but can include massive visceral invasion as well. It has been estimated that about 60% of newly diagnosed prostate cancer patients will have metastases at the time of initial diagnosis (J.I. Epstein et al., Cancer 71:3582-93, 1993).

Surgery or radiotherapy is the treatment of choice for stage A or B prostatic neoplasia. Surgery involves complete removal of the entire prostate (radical prostatectomy), and often removal of the surrounding lymph nodes, lymphadenectomy. Radiotherapy, occasionally used as adjuvant therapy, may be either external or interstitial using ^{125}I. Endocrine therapy is the treatment of choice for more advanced forms. The aim of this therapy is to deprive the prostate cells, and presumably the transformed prostate cells as well, of testosterone. This is accomplished by orchiectomy (castration) or administration of estrogens or synthetic hormones which are agonists of luteinizing hormone-releasing hormone. These cellular messengers directly inhibit testicular and organ synthesis and suppress luteinizing hormone secretion which in turn leads to reduced testosterone secretion by the testes. Despite the advances made in achieving a pharmacologic orchiectomy, the survival rates for those with stage C and D carcinomas are rather bleak. In the short term, the most promising results will be achieved by earlier detection using more sensitive assays.

Yearly rectal examination is very useful for the early detection of prostatic neoplasia. This detection method is fairly simple and straightforward. However, it is subject to bias and not very well standardized. At the earliest, it can only detect stage B carcinoma and has no capacity to determine whether stages C or D are developing. Further, the digital rectal exam is not very sensitive. Approximately 30-60% of men have a prostatic neoplasia that cannot be detected by the physician, which is further complicated by the fact that these men usually present with no symptoms at all. A number of new techniques look promising. These include ultrasound and other methods of noninvasive detection such as magnetic resonance imaging (MRI) with endorectal coil. These methods are limited to the detection of formed tumors and are unable to detect prostatic carcinoma which is just beginning to invade surrounding tissue.

A number of serum antigens have been characterized as markers for prostatic neoplasia. These markers are useful because they are relatively straightforward to assay using noninvasive procedures and may detect prostatic neoplasia at very early stages of development. Both malignant and normal prostate epithelial cells were found to express a prostate-specific acid phosphatase (PAP) which is detectable in serum by biochemical and other immunological techniques. Elevated PAP levels correlate well with neoplasia that has spread beyond the prostate capsule. Consequently, PAP is a useful serum marker for characterizing the later stages of prostatic neoplasia and also for monitoring the progress of the disease in patients.

Various prostate specific antigens have been identified which may be of use in the detection of prostatic carcinoma. One of the more well-studied of these is the prostatic carcinoma associated complex (PAC) also called the glycoprotein complex (G.L. Wright et al., Int. J. Cancer 47:717, 1991). Although specific for prostatic epithelium, this protein complex of 35-310 kD antigens was not correlative for the staging of prostatic carcinoma.

Another valuable prostate marker is the prostate-specific antigen (PSA), a serine protease (kallikrein) found in both normal and neoplastic prostate epithelium. Investigations have determined that there is a direct correlation between serum PSA levels with the size and stage of a tumor. The normal concentration of PSA in men range from 0 to 2.8 ng/ml of serum, using the Hybritech assay, and from 0 to 4.0 ng/ml serum using the Yang assay. In one study, researchers determined that average PSA concentrations in the serum of patients grouped according to severity were proportional to the clinical state of the tumor (T.A. Stamey et al., N. Engl. J. Med. 317:909, 1987). These authors did not indicate whether PSA levels could be used to determine the pathological stage of carcinoma in individual patients. Concentrations of 40 ng/ml were predictive of advanced stages of disease, but the predictive value of serum concentrations of less then 15 ng/ml were less than clear. PSA titers were only marginally useful to distinguish whether the tumor was contained by or had escaped the prostate. Levels greater than 10 ng/ml were typical in patient groups with more advanced and gland-unconfined carcinomas. However, it was not atypical to find high PSA levels in patient groups with gland-confined hyperplasia.

These theories were partly confirmed in a more recent study which looked at serum PSA levels in 209 men with various stages of prostatic neoplasia (J.E. Oesterling et al., J. Urol. 139:766, 1988). These authors determined that PSA levels showed a statistically significant correlation with pathological stages when compared within the various groups. However, the levels were far less useful when looking at patients on an individual basis. There was a large degree of variability between patient groups and a significant number of both false and missed positives. In a rigorous analysis using greater numbers of men and taking into account actual or predicted numbers of carcinoma cells, Partin et al. determined that serum PSA levels were influenced by tumor volume and the stage of differentiation (A.W. Partin et al., J. Urol. 143:747-52, 1990). Mean antigen levels increased with advanced pathological stage, but this seemed to be related more to overall tumor volume than to any particular stage of the disease. In fact, immunohistochemical studies revealed that higher stage tumors actually produced less PSA, possibly due to the diseased state of the cells. The authors concluded that PSA levels are unreliable for preoperative prediction of the pathological stage of individual patients.

Increased public awareness and the development of the serum PSA test has led to an increase in the number of diagnoses of prostate cancer. Consequently, the number of radical prostatectomies has increased dramatically over the past five years in the United States. The operation begins with a thorough sampling of the pelvic lymph nodes. These nodes are reviewed intraoperatively by a pathologist. If prostate cancer is found, indicating that metastatic spread has occurred, the operation is stopped, as only organ-confined prostate cancer can be surgically cured. If the nodes are free of tumor, the prostate is removed.

Recent studies report that 40% to 50% of patients thought to have had disease localized to the prostate with negative lymph nodes and surgical margins, demonstrate a rise in serum PSA by four years after surgery (A.L. Zietman et al., Urology 43:828-33, 1994). These patients were found to be under staged following radical prostatectomy. The most obvious implication is that micrometastases, most likely in the nodes and undetectable by conventional testing including PSA, immunohisto-chemical staining and histopathology, must have been present at the time of surgery. These microscopic foci continue to grow over time, produce PSA, and eventually lead to overt clinical relapse of the cancer.

Advances in molecular biology, specifically the development of the polymerase chain reaction, have made possible very sensitive assays for the detection of specific nucleic acid sequences including specific gene sequences (D.P. Jackson et al., J. Clin. Pathol. 43:499-504, 1990; D.P. Wood et al., J. Histochem. Cytochem. 42:505-11, 1994). These types of studies are dependent, in part, on the inherent stability of nucleic acids, particularly double-stranded DNA. To demonstrate gene expression, a reverse transcription, polymerase chain reaction (RT-PCR) technique must be used to amplify a mRNA signal that is reverse transcribed into cDNA. This is inherently difficult in formalin-fixed, paraffin-embedded archival tissue specimens because of the labile nature of RNA. The ubiquitous presence of endogenous and exogenous RNA degrading enzymes (RNases) further complicates RNA detection.

The feasibility of retrieval of usable quantities of DNA and RNA from archival, paraffin-embedded tissue has recently been demonstrated (R.D. Foss et al., Diag. Mol. Pathol. 3:148-55, 1994). Highly abundant transcripts such as albumin have been reverse transcribed and PCR-amplified from paraffin embedded liver biopsies. RT-PCR assays have also been described for the demonstration of PSA mRNA in circulating blood (A.E. Katz et al., Urology 43:765-75, 1994; and J.G. Moreno et al., Cancer Res. 52:6110-12, 1992), bone marrow (D.P. Wood et al., J. Histochem. Cytochem. 42:505-11, 1994), and lymph nodes (T. Deguchi et al., Cancer Res. 53:5350-54, 1993). However, no assay has been described that can detect micrometastatic PSA mRNA from archival, paraffin-embedded sections.

There are relatively few reports describing the retrieval of usable quantities of any RNA from fixed, paraffin-embedded archival tissues for use in RT-PCR reactions. The majority have amplified relatively abundant genes, including albumin, glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyl transferase, β-actin, retinoblastoma and porphobilinogen (F.V. Weizsacker et al., Biochem. Biophys. Res. Comm. 174:176-80, 1991; J. Ben-Ezra et al., J. Histochem. Cytochem. 39:351-54, 1991; R.D. Foss et al., Mol. Pathol. 3:148-55, 1994). Although RT-PCR assays of prostate cancer cells have been reported to be more sensitive than conventional histopathology and immunologic techniques (T. Deguchi et al., Cancer Res. 53:5350-54, 1993), it has been presumed that rare sequences are lost because RNA undergoes extensive degradation with time.

Several reports have examined the question of nucleotide breakdown with respect to archival tissue (D. Shibata et al., Cancer Res. 48:4564-66, 1988; L. Dubeau et al., Cancer Res. 46:2964-69, 1986). These reports have examined both the breakdown process over time and the effects of various methods of fixation on the subsequent retrieval of both RNA and DNA (D.P. Jackson et al., J. Clin. Pathol. 43:499-504, 1990; and C.E. Greer et al., Am. J. Clin. Path. 95:117-24, 1990). Both DNA and RNA appear to undergo degradation to progressively smaller fragments until lengths less than about 500 bp for DNA, and less than about 250 bp for RNA, have been reached. Pääbo studied DNA retrieved from extremely old biological sources of between 4,000 and 13,000 years old (S. Pääbo, Proc. Natl. Acad. Sci. USA 86:1939-43, 1989). A relatively constant pattern of DNA degradation, characterized by fragments less than 500 bp in length, as well as oxidative damage to pyrimidines and sugar residues was observed.

Evaluation of 40 year old formalin-fixed, paraffin-embedded tissues also demonstrated that short DNA fragments remained, although it was suggested that insufficient neutralization of the formalin in older archival material may lead to acid-induced depurination and subsequent inability of the DNA to function as a template for PCR. Further, formalin is known to react with the amino group of nucleotides to cause cross-linking of DNA with proteins. This process is the basis for formalin's fixative effect, but this alone does not seem to interfere with subsequent amplification by PCR. Several groups have examined the effects of various methods of fixation on subsequent retrieval of nucleic acids for use in PCR amplification schemes. Unfortunately, these studies show wide disagreement between their findings as to the best preservative, indicating that the technique of nucleic acid extraction itself is as important as the method of fixation. For example, using sets of primers designed to yield PCR products ranging in size from 110 bp to 1327 bp, Greer et al. demonstrated preferential amplification of the smaller molecular weight fragments when the source of the DNA was archival (C.E. Greer et al., Am. J. Clin. Pathol. 95:117-24, 1991). Ben-Ezra and coworkers utilized a crude, water-boiled extract for RNA and DNA retrieval (J. Ben-Ezra et al., J. Histochem. Cytochem. 39:351-54, 1991). Foss et al. employed a variation of the RNA retrieval method proposed by Chomczynski and Sacchi (R.D. Foss et al., Mol. Pathol. 3:148-55, 1994; P. Chomczynski et al., Anal. Biochem. 162:156-59, 1987). Further, the time to fixation was quite rapid in all of these studies, whereas it is likely to have been more variable in the majority of stored archival specimens.

A variety of RNA preparative techniques from archival sources have been proposed (D.P. Jackson et al., Lancet 139:1, 1989; G. Stanta et al., BioTechniques 11:304-8, 1991; J. Finke et al., BioTechniques 14:448-53, 1993; R.D. Foss et al., Diag. Mol. Pathol. 3:148-55, 1994). These methods utilize either a digestion by proteinase K, which requires an incubation of up to 18 hours to liberate cellular RNA, or a variation of techniques based on guanidinium isothiocyanate. Based on absorbance of UV light at 260 nm, it was found that the yield of RNA from archival sources to be about one-tenth as much as from fresh tissue. For all practical purposes, a 90% loss of RNA has been considered by those of ordinary skill in the art to be a complete loss of what are typically referred to as the rare sequences.

Summary of the Invention

The present invention overcomes the problems and disadvantages associated with current strategies and designs and provides new methods for the specific detection of rare RNA sequences such as transcription products from metastatic neoplasms, primers useful for the PCR detection of metastases and kits which contain these primers and other reagents useful to rapidly detect metastatic diseases.

One embodiment of the invention is directed to methods for detecting metastatic disorders. A biological sample suspected of containing metastatic tissue is obtained from a patient and, optionally, fixed and attached to a solid support such as paraffin. RNA is extracted from the sample and reverse transcribed to form cDNA. A target sequence of the cDNA, specific to metastatic tissue is amplified, for example, by polymerase chain reaction, and detected. Examples of metastatic disorders which can be detected include disseminated carcinomas of the prostate, breast, lung, liver, pituitary, colorectum, glands, bladder, endometrium, pancreas and cervix, and sarcomas of the muscle, bone, connective tissues and lymph nodes.

Another embodiment of the invention is directed to methods for detecting metastatic prostate carcinoma in a biological sample suspected to contain metastatic prostate tissue. Samples are obtained from patients by surgical excision or post-mortem removal. Nucleic acids are purified from the sample, the RNA reverse transcribed and a resulting target cDNA sequence characteristic for a detectable prostate expression product amplified by polymerase chain reaction. Useful target sequences include sequences which encode PSA, PAC and PAP. Primers which can be utilized to amplify prostate-specific nucleic acids include sequences which correspond to sequences from within exonic regions of the PSA gene.

Another embodiment of the invention is directed to nucleic acid primers which can be used to specifically detect metastatic sequences in a biological sample. Primers contain a sequence corresponding to the exonic regions of the metastatic-specific gene such as, for example, the exonic regions of the prostate specific antigen gene. Primer pairs comprise sequences which are within about 250 nucleotides of each other along an expressed region of the gene.

Another embodiment of the invention is directed to diagnostic kits which can be utilized to detect and to screen biological samples for metastatic-specific sequences in the biological sample. Kits may comprise one or more nucleic acid primers specific for metastatic sequences, a thermostable DNA polymerase for polymerase chain reactions, and other reagents which may be useful for PCR amplification of metastatic sequences from a biological sample.

Other objects and advantages of the invention are set forth in part in the description which follows, and in part, will be obvious from this description, or may be learned from the practice of the invention.

Description of the Drawings

Figure 1	cDNA sequence of the prostate specific antigen gene.
Figure 2	Genomic sequence of the prostate specific antigen gene.
Figure 3	Schematic of the structure of the prostate specific antigen gene with primer positions indicated.
Figure 4	RT-PCR signals from fresh tissues for PSA (lane 2, prostate tissue) and GAPDH (lane 3, human corpus cavernosum).
Figure 5	PCR signals of PSA and GAPDH in dilution sequence of prostate mixed with human corpus cavernosum.
Figure 6	PCR signal for GAPDH from formaldehyde-fixed, rabbit bladder tissue (lane 1) and paraffin-embedded, formaldehyde-fixed, human corpus cavernosum (lane 2), and the negative control (lane 3).
Figure 7	Amplified 247 bp GAPDH sequence in a 2% agarose gel. Lane 1: 123 bp ladder; lanes 2 and 4: HCCSMC cDNA; lanes 3 and 5: human lymph node (with prostate metastasis); and lane 6 minus DNA control.

Figure 8	RT-PCR signals for PSA from prostate tissue using two different primers sets.
Figure 9	Results of primer combinations in RT-PCR experiments with (A) fresh prostate, (B), (C) and (D) paraffin-embedded specimens from different patients.
Figure 10	RT-PCR signals for PSA from archival prostate visualized by ethidium bromide staining.
Figure 11	Southern blot corresponding to ethidium bromide stained gel depicted in Figure 10 demonstrating hybridization of PSA-specific probe to the ethidium-stained bands.

Description of the Invention

As embodied and broadly described herein, the present invention is directed to methods, nucleic acid primers and kits for the detection of rare nucleic acid sequences such as metastatic-specific mRNA sequences in patient-derived biological samples as evidence of metastatic diseases and other disorders.

Detection involves reverse transcription (RT) of RNA sequences obtained from fresh or fixed biological samples and polymerase chain reaction (PCR) amplification of those sequences which are indicative of metastatic invasion of the area sampled. An RT-PCR assay that can be used to detect specific expressed gene sequences in, for example, archival tissues offers several important possibilities. First, such a sensitive assay would allow prostate metastases to be identified retrospectively in sites such as the lymph nodes at levels of sensitivity that exceed conventional pathologic methods. Staging for epidemiological studies would also be greatly improved. Second, as prostate cancer is a very slowly growing tumor, often taking years to manifest clinical change, prospective outcome studies designed to evaluate the effects of gene expression or metastases may require many years to come to fruition. The ability to perform RT-PCR-type experiments on archival tissues allows investigators to perform retrospective outcome studies in much shorter periods of time, since the clinical outcome may already be known. The results of the RT-PCR assay can be evaluated with respect to the clinical outcome. Factors to be examined include serum prostate specific antigen (PSA) levels over time and the relationship of positive assay results to such factors as Gleason grade, clinical stage and preoperative PSA protein levels.

A reliable test also has immediate utility in determining proper and appropriate treatments for patients. Micrometastases, difficult and often impossible to visualize by immunohistochemical or other conventional screening, are detectable using a RT-PCR assay for metastatic-specific sequences. The sensitive and rapid techniques described provide another measure for determining the proper prognosis of a patient. A negative test of the lymph nodes would serve as a powerful prognostic indicator of a good outcome after surgery. Similarly, a positive test definitively indicates that further aggressive treatment will be required after surgery. Lymph nodes are routinely biopsied as part of a surgical procedure and, using the methods of the invention, can now be tested for the presence of tumor metastases at a level of sensitivity that exceeds current histopathology and immunohistochemical techniques. Further, epidemiologic significance of various therapies and treatments can be determined by testing archival lymph node material from patients whose post-operative course over several years is known. Assays that demonstrate a strong correlation to outcome would suggest that in the future, patients with, for example, prostate cancer, should have their lymph nodes screened by this molecular technique prior to undergoing radical procedures such as prostatectomy which are only curative in true, organ-confined diseases.

One embodiment of the invention is directed to a method for detecting a metastatic disorder in a biological sample obtained from a patient. Disorders which can be detected include metastatic neoplasms of any tissue which express a tissue-specific product. Detectable metastases include disseminated carcinoma of the prostate, breast, lung, colorectum, bladder, endometrium, pancreas and cervix. Sarcomas of the muscle, bone, connective tissues and lymph nodes are also identifiable by methods of the invention. Preferably, the metastasis is a micrometastasis which is undetectable by conventional methods of detection such as ultrasound, detection of a tissue-specific antigen in serum, MRI, histochemical staining or morphological observation.

A biological sample suspected of containing metastatic tissue is obtained from a patient. Patients may be any mammal and are preferably humans. Human patients may be male or female adults, children or infants. Samples may also be obtained from a fetus *in utero*. Biological samples are typically obtained by surgical excision from the body such as, for example, biopsy, or during post-mortem examination. Samples may comprise tissue or fluids from any part of the body suspected of containing metastatic tissue either from other signs of disease or simply from routine screening without any outward signs or symptoms of metastatic disease. Tissues which can be routinely screened include the liver and the lymph nodes as these tissues would be expected to be a first site of lymphatic or hematogenous spread of metastatic cells.

Biological samples obtained can be analyzed directly or fixed for storage. Fixation may be by refrigeration at 4°C, freezing at -20°C or lower such as in liquid nitrogen, or by fixation in a fixative. Useful fixatives include solutions containing an alcohol such as ethanol, formalin and Carnoy's formalin, formaldehyde or para-formaldehyde, and commercially packaged fixatives such as Omnifix, Zenker's fixative, Bouin's fixative and B-5. Preferably, the fixed sample may be preserved for long-term storage such as greater than one month, preferably greater than six months and more preferably greater than one year. Sample may also be attached to a solid support. For example, fixed tissue samples are often embedded in oils or waxes such as paraffin. Paraffin embedded samples are often useful because paraffin, as known by those of ordinary skill, can adequately preserve fixed samples for many years. Embedded sample can also be easily handled and manipulated. Portions can be quantitatively removed without affecting the remaining areas of the sample. Alternatively, the entire sample may be analyzed.

Nucleic acids are purified from the fixed sample. Preferably, nucleic acids are extracted from the sample by chemical extraction. Chemicals which are typically used to purify nucleic acids include acid-guanidinium, salts, phenol, chloroform and combinations of salts, phenol and chloroform. Following extraction, nucleic acids are concentrated from an aqueous phase by alcohol precipitation using, for example, isopropyl or ethyl alcohol and high speed centrifugation. The purified nucleic acid contains both RNA and DNA. Due to the prevalence and hardiness of RNases in the environment, RNA in fixed samples is typically considered totally or nearly totally degraded or, in any case, unusable.

Fixation and storage of biological samples severely degrades nucleic acids, and particularly RNA, which may be within those samples. Both DNA and RNA undergo degradation to progressively smaller fragments due to the prevalence and hardiness of nucleases in the environment and as a consequence of necessary manipulations and the fixation treatment. It has not been known, however, whether the breakdown process runs to completion so that no intact nucleic acid remains, or whether the process follows an asymptotic curve, allowing some intact lengths of DNA or RNA to remain for much longer periods of time than previously suspected. In addition, it is also unclear to what degree conventional preservation procedures themselves further degrade the available nucleic acid. Surprisingly, it has been determined that nucleic acids degrade non-randomly in patterns. DNA degrades to contiguous lengths of about 500 base-pairs (bp) and RNA to contiguous lengths of about 250 bp. This length relationship is quite consistent suggesting an asymptotic pattern of degradation. Therefore, certain RNA sequences remain specifically identifiable and can be recovered using the methods of the invention. A series of PCR primers can be designed to yield a variety of amplified lengths. Primers are designed to cross at least one intronic segment of the genome, thus allowing easy distinction between PCR products generated from contaminating genomic DNA and cDNA.

The size of the target chosen for PCR amplification of cDNA derived from archival sources plays a critical role in the success of the reaction. RNA molecules that remain in fixed biological samples are less than about 250 nucleotides in length and in ranges between about 60 nucleotides to about 250 nucleotides and between about 50 nucleotides to about 150 nucleotides, depending on the tissue-type. Consequently, primers designed to produce PCR amplified products (target sequences) of less than about 300 bp are preferred. Preferred sizes of target sequences are between about 50 to about 300 nucleotides in length, more preferably between about 75 to about 250 nucleotides and more preferably between about 100 to about 200 nucleotides. Often, it may be advantageous to use a panel of multiple primer sets, each designed to give a unique product with a unique length, for example, less than about 250 bp. This approach is useful when screening for multiple sequences, any one of which may be indicative of a metastasis. Sensitivity and reproducibility of this type of assay may be improved as at least one of the sets may work even if degradation of the nucleic acids precludes all of the sets from working.

To be detectable and identifiable of metastasis, metastatic cells must express a nucleic acid product which would not otherwise be present in the biological sample. Preferably, there is a known primary tumor and the sample to be analyzed is obtained from a secondary site. For example, a large number of neoplastic metastases contain over expression or various mutations of the p53 gene product, many of which have been extensively studied. A series of primers specific for one or more product expressed from these identifiable genetic mutations can be used to amplify mutated sequences of p53 in the sample. There are also numerous examples of tissue-specific expression products that are indicative of specific types of metastatic diseases.

Prostate carcinoma metastases express a number of products including prostatic carcinoma-associated complex (PAC), prostate-specific acid phosphatase (PAP), and PSA whose mRNA (Figure 3) and genomic sequences (Figure 4) are well-known (P. Schulz et al., Nuc. Acids Res. 16:6226, 1988; H.-G. Klobeck et al., Nuc. Acids Res. 17:3981, 1989). These products, and specifically PSA as indicated in U.S. Patent Nos. 4,446,122 and 4,970,299, and Re. 33,405 which are hereby specifically incorporated by reference, are nearly absolutely specific for prostate cells and detection of PAC, PAP or PSA mRNA in non-prostate tissue is indicative of metastatic prostatic carcinoma. The product of the FAP gene is believed to be specific for metastatic colorectal tissue. FAP sequences in non-colorectal tissue biopsies is strongly indicative of metastatic colorectal carcinoma. The Erb B2 gene product, the estrogen receptor and the progesterone receptor are all specific for breast tissue. Presence of these mRNA sequences in non-breast tissues may be indicative of metastatic mammary carcinoma and particularly when the primary tumor has been demonstrated to express one or more of these products. Additional tissue-specific expression products include insulin and cholecystokinin for pancreatic metastases, albumin for liver metastases, amylase for salivary tumor metastases and luteinizing hormone for pituitary tumor metastases. Tissue-specific expression products identifiable to any metastasized tissue such as carcinomas of the endometrium, bladder, lung or cervix, and sarcomas of the muscle, bone, connective tissues or lymph nodes could be used according to the methods of the invention. Although the absence of such sequences is not necessarily definitive, their presence, if known, will often determine the course of therapy to be administered. In addition, both positive and negative controls can be used to quantitate and to confirm or dismiss any result obtained. As known to those of ordinary skill, a successful prognosis is almost always a requisite for a successful treatment.

Identification of metastatic-specific sequences involves reverse transcription of the RNA sequences in the sample into cDNA. These cDNA sequences are amplified by PCR. PCR amplification of metastatic-specific or target sequences requires the use of specific primers which span protected or undigested regions of the RNA recovered. Protected regions are different for each expression product and should be distinguished from genomic sequences. Distinction of expressed from unexpressed sequences can be accomplished, for example, when the metastatic-specific product is a spliced RNA sequence.

The basic techniques of PCR are described in U.S. Patent Nos. 4,683,195 and 4,683,202, which are hereby specifically incorporated by reference. Variations of these techniques are described in U.S. Patent Nos. 5,043,272, 5,057,410 and 5106,727, which are also specifically incorporated by reference. PCR reactions may be performed using a two or three step method for between about 20 to about 50 cycles and preferably between about 35 to about 45 cycles. Preferably, amplification involves a two-step method with an initial pre-treatment and a final extension. The pre-treatment step is often necessary to eliminate secondary structure in the nucleic acids to allow polymerase reactions to proceed unimpeded. The final extension step elongates those molecules which may have been prematurely terminated prior to complete polymerization. The PCR process comprises an initial pre-treatment of between about 70°C to about 100°C for between about 1 minute to about 30 minutes, followed with between about 20 to about 50 cycles of two or three steps comprising a first step of between about 80°C to about 100°C for between about 30 seconds to about 3 minutes, a second step of between about 45°C to about 75°C for between about 30 seconds to about 3 minutes, and an optional third step of between about 50°C to about 70°C for between about 30 seconds to about 3 minutes, and a final post-treatment of between about 50°C to about 75°C for between about 1 minute to about 15 minutes. A preferred PCR process comprises a pre-treatment of about 94°C for about 15 minutes followed by about 70°C for about 80 seconds, about 39 cycles of a first step of about 94°C for about 80 seconds and a second step of about 70°C for about 80 seconds, and a post-treatment of about 72°C for about six minutes.

The exact reaction conditions to amplify each different metastatic expression product, such as time, temperature, enzyme amounts and the sequence of steps, may vary with primer or target sequence or size, but can be determined empirically by those of ordinary skill in the art using the guidelines of the invention.

Amplified sequences can be easily detected by a number of techniques. For example, sequences may be electrophoresed into an acrylamide or agarose gel and stained with ethidium bromide or another nucleic acid stain. Alternatively, sequences could be transferred to a solid support such as a membrane and stained. Comparison of the bands observed after staining with known molecular weight markers will determine if the amplified sequences correspond to an expected fragment, and thus, indicate the presence of metastatic-specific expression products in the biological sample. Utilizing the methods of the invention, target sequences can be amplified by more than about 100,000 fold, and preferably 1,000,000 fold or more. Another method to measure amplification is to determine the quantity of metastatic tissue present in the sample. It has been determined that metastatic tissue is detectable in the sample at less than one part per 10,000 parts of background, non-metastatic tissue. Such sensitivity has heretofore not been achieved with classical histopathological techniques.

Another embodiment of the invention is directed to the primers which are useful for PCR amplification of rare sequences such as metastatic-specific sequences of biological samples including fresh or fixed tissues. Primers comprise a sequence that corresponds to the exonic regions of the expressed product such that the amplified product detected can be distinguished from amplified genomic sequences. The specific exonic sequences utilized will determine the size of the resulting amplified product. Primers may also comprise sequences such as restriction enzyme or parts of restriction enzyme recognition sites, RNA or DNA polymerase recognition sites, terminal blocking groups and sequences which facilitate the synthesis or manipulation of the primer or the PCR product.

Primers may be between about 10 to about 35 nucleotides in length, preferably between about 15 to about 25 nucleotides and more preferably between about 18 to about 23 nucleotides. Primer pairs should span at least one intronic region and also those regions of the metastatic-specific RNA product which is not degraded by fixation or long-term storage from RNases in the sample. Preferably, the pairs should not hybridize with each other, should not comprise sequences to common regions of the genome and should not possess secondary structure. These features would complicate any results obtained. However, primers with a similar GC content and, consequently, a similar melting temperature, can often be used to optimize the system. Examples of primers which are useful for the detection of PSA nucleic acid sequences are listed in Table 1. It is clear to those of ordinary skill in the art that variations of these sequences such as smaller or larger sequences or adjacent sequences may also be useful.

Table 1
PSA Specific Primers (5'→3')

CGAGA AGCAT TCCCA ACCCT GGC (SEQ ID NO 1)
GGGTG AACTT GCGCA CACAC GTC (SEQ ID NO 2)
CCTGG CCTGT GTCTT CAGGA TG (SEQ ID NO 3)
GAGGT CGTGG CTGGA GTCAT CAC (SEQ ID NO 4)
GTGCT TGTGG CCTCT CGTGG CAG (SEQ ID NO 5)
GGAGG CTCAT ATCGT AGAGC GGG (SEQ ID NO 6)
CTCAC AGCTG CCCAC TGCAT CAG (SEQ ID NO 7)
GTCAT CACCT GGCCT GAGGA ATC (SEQ ID NO 8)

These primers comprise sequences that correspond to various regions of the exons of the prostate specific antigen gene. Complementary primers span regions of the expressed RNA which are not significantly degraded upon fixation or long-term storage of tissue samples. Primer sequences useful for PCR amplification of other metastatic-specific nucleic acids can be determined empirically by those of ordinary skill in the art. Due to the rapidity of the PCR process, the relative ease of generating oligonucleotide primers of any desired sequence, and the ability to conduct multiple PCR experiments simultaneously, such an analysis would be expected to be fairly rapid for any known metastatic-specific nucleic acid (DNA or RNA) expression product.

Another embodiment of the invention is directed to diagnostic kits which can be used to detect and screen for rare or low copy sequences such as metastatic-specific sequences in fixed or fresh biological samples. Kits contain one or more primers that can be used for PCR amplification of metastatic-specific sequences. Kits may also comprise reagents for PCR amplification such as a thermostable DNA polymerase, deoxyoligonucleotides including dATP, dGTP, dCTP and dTTP which may be labeled to facilitate detection, suitable buffers and salts, and other components necessary or useful for amplification and/or detection of amplified sequences. Suitable labels include radionuclides, fluorescent, chemiluminescent or luminescent chemical moieties, digoxenin-dUTP or coupling agents such as biotin, avidin and streptavidin.

Another embodiment of the invention is directed to methods, primers and diagnostic kits which can be used to detect any expressed sequence which is otherwise undetectable in a fresh or fixed biological sample. Such sequences include viral sequences which may be repressed, latent or dormant, sequences representative of lymphoma or other neoplastic disorders and sequences which are indicative of genetic diseases and disorders.

The following examples are offered to illustrate embodiment of the present invention, but should not be viewed as limiting the scope of the invention.

Examples

Example 1 Sample Preparation and RNA Extraction.

Nucleic acid was extracted from archival, formalin-fixed, paraffin-embedded tissue specimens of human prostate tissue, formalin-fixed rabbit bladder tissue and formalin-fixed human corpus cavernosa tissue, a non-PSA secreting genitourinary tract tissue. Paraffin-embedded samples were trimmed of excess paraffin and weighed. Samples were placed into a petri dish and finely minced with a razor blade. Paraffin was removed from the tissue by two incubations of five minutes each in xylene at 55°C. Total RNA for all samples was isolated by grinding the samples for several minutes with a tissue homogenizer (Janke and Kunkel IKA Labortechnik Ultra-Turrax T25) at a maximum speed of 24,000 rpm in the presence of TRI-reagent (a rapid acid-guanidinium technique) (Molecular Research Center, Inc.; Cincinnati, OH). To prevent sample carry over, the homogenizer probe was cleaned by multiple, sequential washes with 100% ethanol and distilled water. A further wash with 0.1N NaOH may also be used to ensure complete destruction of all nucleic acid sequences. The homogenate was allowed to stand for 5 minutes at room temperature to dissociate nucleoprotein complexes. Following the addition of one-fifth volume chloroform, samples were centrifuged at 3,000 x g for 30 minutes. RNA was precipitated from the aqueous phase using isopropanol and glycogen (20 μg) as carrier by rapidly cooling samples to -80°C. RNA precipitates were centrifuged at 3,000 x g for 20 minutes at -4°C to obtain pellets which were subsequently washed in 70% ethanol, resuspended in 500 μl of H_20 treated with diethylpyrocarbonate (DEPC) and further purified with a phenol-chloroform extraction. Samples were again precipitated with ethanol and ammonium acetate using glycogen (20 μg) as a carrier. RNA yield was calculated by measuring absorbance of UV at 260 nm. In most cases, the final RNA pellet was washed with 70% ethanol, dried in a vacuum centrifuge, and used in the reverse transcription reaction. RNA obtained was sampled and the samples separated on a denaturing agarose gel and visualized after staining with ethidium bromide. No visible evidence of 18s or 28s ribosomal fragments was detected indicating that at least partial degradation had occurred.

Example 2 Reverse Transcription Reactions.

RNA pellets were incubated in a 50 μl of a solution containing 25 mM Tris-HCl, 37.5 mM KCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM each of dATP, dCTP, dGTP and dTTP, and 1 μM random hexamers of DNA

(Perkin-Elmer Cetus; Norwalk, CT; and Roche Molecular Systems; Branchburg, NJ). These varied sequences are more likely to bind to degraded RNA than either oligo dT's or specific downstream primers, both of which require an intact recognition sequence. In addition, this generates a mixed cDNA population, allowing multiple RT-PCR reactions involving a variety of mRNA transcripts to be studied. The mixture was heated to 65°C for 15 minutes, quickly cooled to 4°C and rewarmed to room temperature. Two hundred units of murine Moloney leukemia virus (MMLV) reverse transcriptase (GIBCO/BRL; Grand Island, NY) and 40 units of RNasin. RNase inhibitor (Promega; Madison, WI) were added to each tube. Incubation was performed in a step wise fashion to maximize cDNA yield in a programmable aluminum block thermocycler (MJ Research; Watertown, MA) programmed as follows: Step 1: 15°C for 2 minutes; Step 2: 17°C for 2 minutes; Step 3: 19°C for 2 minutes; Step 4: 21°C for 2 minutes; Step 5: 23°C for 2 minutes; Step 6: 25°C for 2 minutes; Step 7: 27°C for 2 minutes; Step 8: 29°C for 2 minutes; Step 9: 32°C for 2 minutes; Step 10: 37°C for 60 minutes; and Step 11: 42°C for 30 minutes to complete synthesis, followed by inactivation of the reverse transcriptase by heating to 70°C for 5 minutes.

Example 3 Polymerase Chain Reactions.

A 2.5 μl sample of the reverse transcription reaction was used for amplification with PCR in a total volume of 50 μl prepared with final concentrations as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.2 mM each dNTP. In each individual experiment, primer sets were chosen to give various PCR product lengths, with a final concentration of 0.2 mM. Five units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus; Norwalk, CT; and Roche Molecular Systems; Branchburg, NJ) were added to the reaction. Primer sequences and relative locations, and the lengths of all pair combinations are shown in Figure 3 and in Tables 2 and 3.

Table 2
Sequences for PSA Primers

Primer	Sequence (5'→3')	cDNA	gDNA	length
SEQ ID NO 1	CGAGAAGCATTCCCAACCCTGGC	93-115	1734-1756	23
SEQ ID NO 2	GGGTGAACTTGCGCACACACGTC	544-566	3957-3979	23
SEQ ID NO 3	CCTGGCCTGTGTCTTCAGGATG	244-265	3514-3535	22
SEQ ID NO 4	GAGGTCGTGGCTGGAGTCATCAC	341-363	3611-3632	23
SEQ ID NO 5	GTGCTTGTGGCCTCTCGTGGCAG	118-140	1759-1781	23
SEQ ID NO 6	GGAGGCTCATATCGTAGAGCGGG	294-316	3564-3586	23
SEQ ID NO 7	CTCACAGCTGCCCACTGCATCAG	181-203	1822-1844	23
SEQ ID NO 8	GTCATCACCTGGCCTGAGGAATC	326-348	3596-3618	23

PSA probe sequence:5'-GGAACAAAAGCGTGATCTTGCTGGG-3' (SEQ ID NO 9)

cDNA(bp) = complementary DNA base pair length.
gDNA(bp) = genomic DNA base pair length.

Table 3
Predicted product lengths of various PCR primer combinations

		Upstream Primer		
		1	5	7
Downstream Primer	2:	473	449	386
	3:	173	148	85
	4:	271	246	183
	6:	224	199	136
	8:	256	231	168

Reactants were overlayed with 50 µl mineral oil and subjected to a 2-step regimen of temperature cycling in the aluminum block thermocycler programmed as follows: pre-treatment: 94°C for 15 minutes followed with 70°C for 80 seconds; treatment: 39 cycles of 94°C for 80 seconds and 70°C for 80 seconds; post-treatment: final extension step of 72°C for 6 minutes. PCR products formed were analyzed by ethidium bromide staining after electrophoresis in 2.0% agarose in 89 mM Tris, 89 mM borate and 0.2 mM EDTA (TBE buffer). Bands were visualized with ultraviolet light.

Example 4 Southern Blotting and Detection of Metastatic Sequences.

After agarose gel electrophoresis of the PCR products, the DNA was transferred to Duralose, a nylon reinforced nitrocellulose membrane (Stratagene, La Jolla, CA), by Posiblot, a positive pressure blotting apparatus (Stratagene; La Jolla, CA). A 363 bp PSA cDNA probe (nucleotides 1351-3979 in genomic DNA (gDNA) PSA and nucleotides 203-566 in cDNA PSA) was amplified from human prostate cDNA derived from liquid nitrogen frozen specimens at the time of radical prostectomy using the following primers:

upstream primer = 5'-GGAACAAAAGCGTGATCTTGCTGGG-3' (SEQ ID NO 9)

downstream primer = 5'-GGGTGAACTTGCGCACACACGTC-3' (SEQ ID NO 10)

Products amplified were purified on a 2% low melting temperature agarose gel (GIBCO/BRL; Grand Island, NY) run in 50 mM Tris-acetate, 0.2 mM EDTA, pH 8.0, buffer. A 363 bp band was excised and the nucleic acid recovered by melting the fragment at 65°C and extracting with an equal volume of phenol followed by an equal volume of chloroform precipitated with ethanol in the presence of 2.5 M ammonium acetate. Labeled DNA fragments were dissolved in 10 mM Tris-HCl, 1.0 mM EDTA, pH 7.4, at 100 ng/µl and labeled by random-primed DNA synthesis using the T7 DNA polymerase, Quickprime (Pharmacia Biotech; Piscataway, NJ) and α^{32}P-dCTP at 800 Ci/mm (Du Pont NEN Products; Boston, MA). The PSA probe as well as sheared salmon sperm DNA were denatured by heating to 100°C for 2 minutes prior to use. Blots were prehybridized in 15 ml of Quikhyb (Stratagene; La Jolla, CA) and 150 µl of salmon sperm DNA (10 mg/ml) at 68°C for 2 hours. Blots were washed under low stringency (2 x SSC, 0.1% SDS at 25°C) and high stringency (0.2 x SSC, 0.1% SDS at 60°C), and imaged by autoradiography.

Example 5 Validation of PCR Assay for PSA and GAPDH.

To validate use of the PCR reaction, a pair of universal primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed to amplify a 247 bp fragment in most mammals. There is 100% sequence identity in the rat and the human sequences in the primers (J. Tso et al., Nuc. Acids Res. 13:2485-502, 1985). This transcript was chosen because of its abundance and presence in most cell types. RNA prepared from snap frozen prostate biopsies, and human corpus cavernosum smooth muscle cells (HCCSMC) was reverse transcribed and amplified by 2-step PCR. HCCSMC was chosen as a representative human tissue of genitourinary tract origin that does not produce PSA and therefore serves as a positive control for GAPDH expression and a negative control for PSA expression. Expected bands sizes are listed in Table 3.

For both sets of cDNAs, PSA and GAPDH primers were used in the same mixture, with 40 cycles of 94°C for 1 minute, 72°C for 1 minute, with a final 6 minute extension at 72°C. Figure 4 depicts the results of a 2% agarose gel as follows: lane 1: BRL 123 bp ladder molecular weight markers, lane 2: HCCSMC cDNA, and lane 3: prostate cDNA. In all cases, at least one intronic region is crossed to distinguish between amplification of genomic DNA and amplification of cDNA by product size. Both HCCSMC and prostate show the presence of a 247 bp GAPDH amplified fragment while only the prostate sample has a PSA amplified band (473 bp). Other experiments have been done to optimize PCR conditions and validate primers with only one primer set in the reaction. These results indicate that both primer sets work in a 2-step PCR assay, and that PSA primers give positive results in prostate, but not in negative controls.

Example 6 Sensitivity and Limits of RT-PCR From Archival Specimens.

The sensitivity of the assay was determined by diluting HCCSMC cDNA with a known amount of prostate cDNA. Amplified sequence were visualized on a 2% agarose gel in 1 x TBE buffer after ethidium staining. As shown in Figure 5, lane 1: 123 bp ladder molecular weight markers; lane 2: 1:1 mixture of prostate cDNA and HCCSMC cDNA; lane 3: 1:10 mixture; lane 4: 1:100; lane 5 1:1000; lane 6: 1:10,000; lane 7: primer control (cDNA); and lane 8: HCCSMC alone. After 40 cycles of 2-step PCR, a 1:10,000 dilution of prostate cDNA with HCCSMC cDNA yielded a detectable ethidium bromide stained band. Using nonisotopic detection, as described below, detection of one sequence in 10^6 could possibly be achieved.

RNA was prepared from formaldehyde-fixed rabbit bladder, and paraffin-embedded blocks of human corpus cavernosum, and human lymph node with a prostate tumor metastasis. RT-PCR was then performed on these samples to amplify a 247 bp GAPDH DNA fragment. Figure 6 shows formaldehyde fixed rabbit bladder (lane 1), paraffin-embedded human corpus cavernosum (lane 2), and minus cDNA control (lane 3). Each lane contains primers 1 and 2, designed to amplify a 473 bp PCR product, as well as a primers designed to amplify a 247 bp fragment from GAPDH:

upstream primer = 5'-ACTGGCGTCTTCACCACCATGG-3' (SEQ ID NO 11)

downstream primer = 5'-GAGTACTGGTGTCAGGTACGGT-3' (SEQ ID NO 12)

These data indicate that while the RNA in archival blocks may be partially degraded, PCR amplifiable material is obtained by using random hexamers to prepare cDNA and an appropriate target size for PCR. Based on preliminary data for PCR of PSA from a known prostate tumor metastasis, the 473 bp target size may be too large, as minimal amplification was detected on ethidium bromide stained gels. However, the detection of 247 bp GAPDH from the same sample suggests that amplifiable RNA is present, but in smaller fragments. Figure 7 shows a 2% agarose gel representing: lane 1: 123 bp ladder, lanes 2 and 4: HCCSMC cDNA, lanes 3 and 5: human lymph node (with prostate metastasis) cDNA, and lane 6: minus DNA control. It should be noted that only 10% of the cDNA was used for the amplifications, and also that all detection in these preliminary experiments has relied on ethidium bromide stained gels. Nonisotopic detection such as Southern blots detected with luminescent probes, will further enhance the sensitivity of the assay. Therefore, by determining the optimum size of amplifiable fragments, this assay should detect PSA in micrometastases in archival lymph node specimens.

Example 7 RT-PCR Assay for the Expression of PSA.

The assay was designed with four criteria: (1) that cDNA and genomic DNA amplification products would have unique, distinguishable sizes, (2) that all primers anneal at 72°C(±4°C) such that primer annealing and elongation by Taq polymerase could take place in the same step, (3) that all primers be about the same length and have about the same GC content, and (4) that the optimal target size of the RNA fragments be determined such that nested primers could later be used for secondary amplification. Table 1 lists eight primers that were used. By using the second, third, and fourth exons of the PSA gene, the large intervening sequences were easy to distinguish (signal only from prostatic derived tissues) from any contaminating genomic DNA (signal in all cells). The optimal target size for PCR amplification of PSA cDNA was determined using archival paraffin-embedded prostate cDNA with the primer sets of Table 2. Results are shown in Figure 8: lane 1, BRL 123 bp ladder; lane 2, primers 1 and 4 and a 271 bp product; lane 3, primers 3 and 5 and a 148 bp product; and lane 4, primers 3 and 7 and an 85 bp product. Note that there is a size at which amplification is enhanced (148 bp) suggesting that the degraded RNA in the archival tissue presents an optimal target size as suggested above. The range to be examined is 271 bp to 85 bp in approximately 25 bp increments. The feasibility of this assay was further tested by using formalin-fixed, paraffin-embedded prostate tissue to amplify PSA using the primers described in the above proposal. Primer sets 1 and 4 (271 bp product), 3 and 5 (148 bp product), and 3 and 7 (85 bp product) were used on cDNA prepared from 80 mg of paraffin-embedded prostate. Total cDNA in each reaction was the equivalent of that from 8 mg of tissue.

Example 8 RT-PCR Assay of Frozen Prostate Tissue.

To test the validity and specificity of the PSA primers, the RT-PCR assay was performed on RNA prepared from freshly frozen prostate specimens removed at operation and frozen immediately in liquid nitrogen. Figure 9, panel A, depicts an ethidium bromide stained 2.0 % agarose gels in 1 x TBE buffer after electrophoretic separation. M = 123 bp ladder, lane 1 is stock concentration of cDNA derived from fresh prostate tissue. Panel A is a test of the various primer pairs on fresh human prostate tissue. Panels B, C and D are tests of these same primers on formalin-fixed, paraffin-embedded human prostate tissues. Unique bands were visualized at each of the predicted target lengths for all of the specified primer combinations. Additional bands at higher molecular weights may indicate non-specific amplification by DNA polymerase or potential amplification of genomic DNA (note constant high molecular weight band in essentially all lanes). The design of the primers was such that the large intervening intron 2 between most of the primer sets (except primer 2) allows easy distinction between amplification of cDNA and genomic DNA. Amplification of the genomic DNA should yield products that are at least 1630 bp longer than the corresponding cDNA with the same set of primers. For primer sets where primer 2 is the downstream primer, the PCR product from the genomic DNA should be at least 1872 bp longer than the product from the corresponding cDNA, reflecting the inclusion of both introns 1 and 2.

In dilution studies using prostate cDNA prepared from frozen tissue diluted into a background of human corpus cavernosum cDNA, a non-PSA secreting genitourinary tract tissue, it was found that the assay was able to demonstrate a signal from the prostate at dilutions of 1:10,000 when analyzed by ethidium bromide staining.

Example 9 RT-PCR Assay of Formalin-Fixed Prostate Tissue.

Three tested specimens of archival, formalin-fixed, paraffin--embedded tissue are shown in Figure 9, panels B, C and D, as ethidium-bromide stained gels. In all three of the specimens tested, PCR products were obtained using primers that generated relatively low molecular weight fragments in the range of 85 to 246 bp in length when analyzed by ethidium bromide staining after electrophoresis. The primer sets designed to generate fragments with lengths of 85, 148, 173, 183 and 246 bp worked particularly well, although there were multiple other bands present besides the 183 bp product in that lane in all samples tested. PCR products were rarely visualized when the primers were designed to give fragments longer than 248 bp in length.

Figure 10 depicts an ethidium stained gel of prostate from the same patient as used in Figure 9, panel B. Each successive lane represents a ten-fold dilution of cDNA prior to PCR amplification. A Southern blot of same gel demonstrates that the bands which could be visualized by ethidium bromide in Figure 10, would also hybridized to a radioactive PSA-specific probe further confirming the identity of the amplified sequences (Figure 11). These results suggests that RNA degradation most likely occurs during the phase of tissue preservation and progresses to yield fragments that are less than 250 bp in length. Interestingly, not all of the primer sets designed to yield PCR products less than 250 bp do so, most likely as a result of sub-optimal conditions for that particular reaction. In only one tissue block was a 473 bp product seen. This tissue was only a few weeks old, suggesting that degradation had not progressed as completely as in the other two specimens. It is less likely that a particular primer did not function adequately, since the tested sets were redundant with respect to any one primer. Also, all of the primers worked well in reactions involving fresh tissue, making the likelihood of complete primer failure less likely.

Example 10 Detection of RNA in Paraffin-Embedded Lymph Nodes.

RNA was prepared from archival, formalin-fixed paraffin-embedded lymph nodes from a patient who had undergone pelvic lymphadenectomy and radical prostatectomy several years prior. At the time of the original operation, lymph nodes were evaluated by conventional histopathology and judged to be free of prostate metastases. The specimen was tested using the RT-PCR procedure described in Examples 2 and 3. PSA mRNA were detected in amplification reactions using five of the primer sets of Table 3.

Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. The specification and examples should be considered exemplary only with the true scope and spirit of the invention indicated by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: EDELSTEIN, Robert A.
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(ii) TITLE OF THE INVENTION: PRIMER FOR THE PCR AMPLIFICATION OF METASTATIC SEQUENCES

(iii) NUMBER OF SEQUENCES: 19

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/368,706
(B) FILING DATE: 04-JAN-1995
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(A) APPLICATION NUMBER:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGAGAAGCAT TCCCAACCCT GGC 23

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGTGAACTT GCGCACACAC GTC 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTGGCCTGT GTCTTCAGGA TG 22

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGGTCGTGG CTGGAGTCAT CAC 23

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTGCTTGTGG CCTCTCGTGG CAG 23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAGGCTCAT ATCGTAGAGC GGG 23

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTCACAGCTG CCCACTGCAT CAG 23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCATCACCT GGCCTGAGGA ATC 23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGAACAAAAG CGTGATCTTG CTGGG 25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGTGAACTT GCGCACACAC GTC 23

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTGGCGTCT TCACCACCAT GG 22

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGTACTGGT GTCAGGTACG GT 22

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1729 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
AAGCTTTCCC TTCTCCCAGT CCAAGACCCC AAATCACCAC AAAGGACCCA ATCCCCAGAC      60
TCAAGATATG GTCTGGGCGC TGTCTTGTGT CTCCTACCCT GATCCCTGGG TTCAACTCTG     120
CTCCCAGAGC ATGAAGCCTC TCCACCAGCA CAGCCACCAA CCTGCAAACC TAGGGAAGAT     180
TGACAGAATT CCCAGCCTTT CCCAGCTCCC CCTGCCCATG TCCCAGGACT CCCAGCCTTG     240
GTTCTCTGCC CCCGTGTCTT TTCAAACCCA CATCCTAAAT CCATCTCCTA TCCGAGTCCC     300
CCAGTTCCTC CTGTCAACCC TGATTCCCCT GATCTAGCAC CCCCTCTGCA GGTGCTGCAC     360
CCCTCATCCT GTCTCGGATT GTGGGAGGCT GGGAGTGCGA GAAGCATTCC CAACCCTGGC     420
AGGTGCTTGT AGCCTCTCGT GGCAGGGCAG TCTGCGGCGG TGTTCTGGTG CACCCCCAGT     480
GGGTCCTCAC AGCTACCCAC TGCATCAGGA ACAAAAGCGT GATCTTGCTG GGTCGGCACA     540
GCCTGTTTCA TCCTGAAGAC ACAGGCCAGG TATTTCAGGT CAGCCACAGC TTCCCACACC     600
CGCTCTACGA TATGAGCCTC CTGAAGAATC GATTCCTCAG GCCAGGTGAT GACTCCAGCC     660
ACGACCTCAT GCTGCTCCGC CTGTCAGAGC CTGCCGAGCT CACGGATGCT ATGAAGGTCA     720
TGGACCTGCC CACCCAGGAG CCAGCACTGG GGGACCACCT GCTACGCCTC AGGCTGGGGC     780
AGCATTGAAC CAGAGGAGTT CTTGACCCCA AAGAAACTTC AGTGTGTGGA CCTCCATGTT     840
ATTTCCAATG ACGTGTGTGC GCAAGTTCAC CCTCAGAAGG TGACCAAGTT CATGCTGTGT     900
GCTGGACGCT GGACAGGGGG CAAAAGCACC TGCTCGGGTG ATTCTGGGGG CCCACTTGTC     960
TGTAATGGTG TGCTTCAAGG TATCACGTCA TGGGGCAGTG AACCATGTGC CCTGCCCGAA    1020
AGGCCTTCCC TGTACACCAA GGTGGTGCAT TACCGGAAGT GGATCAAGGA CACCATCGTG    1080
GCCAACCCCT GAGCACCCCT ATCAACTCCC TATTGTAGTA AACTTGGAAC CTTGGAAATG    1140
ACCAGGCCAA GACTCAGGCC TCCCCAGTTC TACTGACCTT TGTCCTTAGG TGTGAGGTCC    1200
AGGGTTGCTA GGAAAAGAAA TCAGCAGACA CAGGTGTAGA CCAGAGTGTT TCTTAAATGG    1260
TGTAATTTTG TCCTCTCTGT GTCCTGGGGA ATACTGGCCA TGCCTGGAGA CATATCACTC    1320
AATTTCTCTG AGGACACAGA TAGGATGGGG TGTCTGTGTT ATTTGTGGGG TACAGAGATG    1380
AAAGAGGGGT GGGATCCACA CTGAGAGAGT GGAGAGTGAC ATGTGCTGGA CACTGTCCAT    1440
GAAGCACTGA GCAGAAGCTG GAGGCACAAC GCACCAGACA CTCACAGCAA GGATGGAGCT    1500
GAAAACATAA CCCACTCTGT CCTGGAGGCA CTGGGAAGCC TAGAGAAGGC TGTGAACCAA    1560
GGAGGGAGGG TCTTCCTTTG GCATGGGATG GGGATGAAGT AAGGAGAGGG ACTGACCCCT    1620
GGAAGCTGAT TCACTATGGG GGGAGGTGTA TTGAAGTCCT CCAGACAACC CTCAGATTTG    1680
ATGATTTCCT AGTAGAACTC ACAGAAATAA AGAGCTGTTA TACTGTGAA                1729
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 1728 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
GGTGTCTTAG GCACACTGGT CTTGGAGTGC AAAGGATCTA GGCACGTGAG GCTTTGTATG      60
```

```
AAGAATCGGG GATCGTACCC ACCCCCTGTT TCTGTTTCAT CCTGGGCATG TCTCCTCTGC    120
CTTTGTCCCC TAGATGAAGT CTCCATGAGC TACAAGGGCC TGGTGCATCC AGGGTGATCT    180
AGTAATTGCA GAACAGCAAG TGCTAGCTCT CCCTCCCCTT CCACAGCTCT GGGTGTGGGA    240
GGGGGTTGTC CAGCCTCCAG CAGCATGGGG AGGGCCTTGG TCAGCCTCTG GGTGCCAGCA    300
GGGCAGGGGC GGAGTCCTGG GGAATGAAGG TTTTATAGGG CTCCTGGGGG AGGCTCCCCA    360
GCCCCAAGCT TACCACCTGC ACCCGGAGAG CTGTGTCACC ATGTGGGTCC CGGTTGTCTT    420
CCTCACCCTG TCCGTGACGT GGATTGGTGA GAGGGGCCAT GGTTGGGGGG ATGCAGGAGA    480
GGGAGCCAGC CCTGACTGTC AAGCTGAGGC TCTTTCCCCC CCAACCCAGC ACCCCAGCCC    540
AGACAGGGAG CTGGGCTCTT TTCTGTCTCT CCCAGCCCCA CTTCAAGCCC ATACCCCCAG    600
TCCCCTCCAT ATTGCAACAG TCCTCACTCC CACACCAGGT CCCCGCTCCC TCCCACTTAC    660
CCCAGAACTT TCTTCCCATT TGCCCAGCCA GCTCCCTGCT CCCAGCTGCT TTACTAAAGG    720
GGAAGTTCCT GGGCATCTCC GTGTTTCTCT TTGTGGGGCT CAAAACCTCC AAGGACCTCT    780
CTCAATGCCA TTGGTTCCTT GGACCGTATC ACTGGTCCAT CTCCTGAGCC CCTCAATCCT    840
ATCACAGTCT ACTGACTTTT CCCATTCAGC TGTGAGTGTC CAACCCTATC CCAGAGACCT    900
TGATGCTTGG CCTCCCAATC TTGCCCTAGG ATACCCAGAT GCCAACCAGA CACCTCCTTC    960
TTTCCTAGCC AGGCTATCTG GCCTGAGACA ACAAATGGGT CCCTCAGTCT GGCAATGGGA   1020
CTCTGAGAAC TCCTCATTCC CTGACTCTTA GCCCCAGACT CTTCATTCAG TGGCCCACAT   1080
TTTCCTTAGG AAAAACATGA GCATCCCCAG CCACAACTGC CAGCTCTCTG AGTCCCCAAA   1140
TCTGCATCCT TTTCAAAACC TAAAAACAAA AAGAAAAACA AATAAAACAA AACCAACTCA   1200
GACCAGAACT GTTTTCTCAA CCTGGGACTT CCTAAACTTT CCAAAACCTT CCTCTTCCAG   1260
CAACTGAACC TCGCCATAAG GCACTTATCC CTGGTTCCTA GCACCCCTTA TCCCCTCAGA   1320
ATCCACAACT TGTACCAAGT TTCCCTTCTC CCAGTCCAAG ACCCCAAATC ACCACAAAGG   1380
ACCCAATCCC CAGACTCAAG ATATGGTCTG GGCGCTGTCT TGTGTCTCCT ACCCTGATCC   1440
CTGGGTTCAA CTCTGCTCCC AGAGCATGAA GCCTCTCCAC CAGCACCAGC CACCAACCTG   1500
CAAACCTAGG CAAGATTGAC AGAATTCCCA GCCTTTCCCA GCTCCCCCTG CCCATGTCCC   1560
AGGACTCCCA GCCTTGGTTC TCTGCCCCCG TGTCTTTTCA AACCCACATC CTAAATCCAT   1620
CTCCTATCCG AGTCCCCCAG TTCCCCCTGT CAACCCTGAT TCCCCTGATC TAGCACCCCC   1680
TCTGCAGGCG CTGCGCCCCT CATCCTGTCT CGGATTGTGG GAGGCTGG                1728
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Val Trp Ala Leu
 1              5
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 245 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Ala Ala Pro Leu Ile Leu Ser Arg Ile Val Gly Gly Trp Glu Cys Glu
1               5                   10                  15
Lys His Ser Gln Pro Trp Gln Val Leu Val Ala Ser Arg Gly Arg Ala
            20                  25                  30
Val Cys Gly Gly Val Leu Val His Pro Gln Trp Val Leu Thr Ala Thr
        35                  40                  45
His Cys Ile Arg Asn Lys Ser Val Ile Leu Leu Gly Arg His Ser Leu
    50                  55                  60
Phe His Pro Glu Asp Thr Gly Gln Val Phe Gln Val Ser His Ser Phe
65                  70                  75                  80
Pro His Pro Leu Tyr Asp Met Ser Leu Leu Lys Asn Arg Phe Leu Arg
                85                  90                  95
Pro Gly Asp Asp Ser Ser His Asp Leu Met Leu Leu Arg Leu Ser Glu
            100                 105                 110
Pro Ala Glu Leu Thr Asp Ala Met Lys Val Met Asp Leu Pro Thr Gln
        115                 120                 125
Glu Pro Ala Leu Gly Thr Thr Cys Tyr Ala Ser Gly Trp Gly Ser Ile
    130                 135                 140
Glu Pro Glu Glu Phe Leu Thr Pro Lys Lys Leu Gln Cys Val Asp Leu
145                 150                 155                 160
His Val Ile Ser Asn Asp Val Cys Ala Gln Val His Pro Gln Lys Val
                165                 170                 175
Thr Lys Phe Met Leu Cys Ala Gly Arg Trp Thr Gly Gly Lys Ser Thr
            180                 185                 190
Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Cys Asn Gly Val Leu Gln
        195                 200                 205
Gly Ile Thr Ser Trp Gly Ser Glu Pro Cys Ala Leu Pro Glu Arg Pro
    210                 215                 220
Ser Leu Tyr Thr Lys Val Val His Tyr Arg Lys Trp Ile Lys Asp Thr
225                 230                 235                 240
Ile Val Ala Asn Pro
                245
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 7 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Leu Leu Tyr Asp Gln Met
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 16 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Trp Val Pro Val Val Phe Leu Thr Leu Ser Val Thr Trp Ile Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 13 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: N-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ala Ala Pro Leu Ile Leu Ser Arg Ile Val Gly Gly Trp
1 5 10

We Claim:

1. A method for detecting a metastatic disorder comprising the steps of:
    a) providing a fixed biological sample suspected of containing metastatic tissue;
    b) reverse transcribing RNA contained in the fixed sample to cDNA;
    c) amplifying a target sequence of the cDNA wherein said target sequence is specific to said metastatic tissue; and
    d) detecting the amplified target sequence.

2. The method of claim 1 wherein the metastasis detected is selected from the group consisting of metastatic carcinomas of the prostate, breast, colorectum, liver, pancreas, pituitary, endometrium, bladder, lung and cervix.

3. The method of claim 1 wherein the fixed biological sample is a surgically removed sample of tissue.

4. The method of claim 3 wherein the tissue sample is human lymphatic tissue.

5. The method of claim 1 wherein the sample is fixed by treating the sample with a formalin solution or a formaldehyde solution, or by freezing.

6. The method of claim 1 wherein the fixed sample can be stored for greater than one year.

7. The method of claim 1 wherein the fixed biological sample is embedded in a solid support.

8. The method of claim 7 wherein the solid support is paraffin.

9. The method of claim 1 wherein the target sequence is amplified by polymerase chain reaction.

10. The method of claim 9 wherein the polymerase chain reaction comprises a pre-treatment of about 94°C for about 15 minutes followed by about 70°C for about 80 seconds, about 39 cycles of a first step of about 94°C for about 80 seconds and a second step of about 70°C for about 80 seconds, and a post-treatment of about 72°C for about six minutes.

11. The method of claim 9 wherein the polymerase chain reaction is a two-step reaction.

12. The method of claim 9 wherein the polymerase chain reaction is performed for between about 20 to about 50 cycles.

13. The method of claim 1 wherein the target sequence is amplified by more than about 1,000,000 fold.

14. The method of claim 1 wherein the metastatic tissue is present in the sample at about one part per 10,000 parts of non-metastatic tissue.

15. The method of claim 1 wherein the target sequence is selected from the group consisting of DNA sequences which correspond to mRNA of prostate specific antigen, prostate-specific acid phosphatase, prostatic carcinoma-associated complex, Erb B2, p53, albumin, progesterone and estrogen receptor, FAP, amylase, luteinizing hormone and insulin.

16. The method of claim 1 wherein the target sequence is less than about 250 nucleotides in length.

17. The method of claim 1 wherein the amplified target sequence is detected isotopically.

18. A method for detecting metastatic prostate carcinoma comprising the steps of:

a) providing a fixed biological sample suspected to contain metastatic prostate tissue;

b) reverse transcribing RNA of the fixed sample to cDNA;

c) PCR amplifying a target sequence of the cDNA wherein said target sequence is specifically expressed in said prostate tissue; and

d) detecting the amplified sequence.

19. The method of claim 18 wherein the fixed biological sample is a surgically removed sample of human lymphatic tissue.

20. The method of claim 18 wherein the sample is fixed by treating the sample with a formalin solution or a formaldehyde solution, or by freezing.

21. The method of claim 18 wherein the fixed sample is embedded in paraffin.

22. The method of claim 18 wherein the target sequence corresponds to a sequence of prostate specific antigen mRNA.

23. The method of claim 18 wherein the target sequence is less than about 250 nucleotides in length.

24. The method of claim 18 wherein PCR amplification is performed with a primer comprising a sequence selected from the group consisting of:

5'-CGAGAAGCATTCCCAACCCTGGC-3' (SEQ ID NO 1);
5'-GGGTGAACTTGCGCACACACGTC-3' (SEQ ID NO 2);
5'-CCTGGCCTGTGTCTTCAGGATG-3' (SEQ ID NO 3);
5'-GAGGTCGTGGCTGGAGTCATCAC-3' (SEQ ID NO 4);
5'-GTGCTTGTGGCCTCTCGTGGCAG-3' (SEQ ID NO 5);
5'-GGAGGCTCATATCGTAGAGCGGG-3' (SEQ ID NO 6);
5'-CTCACAGCTGCCCACTGCATCAG-3' (SEQ ID NO 7); and
5'-GTCATCACCTGGCCTGAGGAATC-3' (SEQ ID NO 8).

25. The method of claim 18 wherein the prostate tissue is present in the sample at less than about one part per 10,000 parts of non-prostate tissue.

26. The method of claim 18 wherein the target sequence is amplified by more than about 10^6 fold.

27. A pair of primers for PCR amplification of a nucleic acid expressed from a metastatic-specific gene wherein a sequence of one primer corresponds to an exonic sequence of said gene and a sequence of the other primer corresponds to a sequence of a different exonic sequence of said gene wherein said sequences are within about 250 nucleotides of each other along an expressed region of said gene.

28. The primers of claim 27 wherein the metastatic-specific gene is selected from the group consisting of the genes which encode prostate specific antigen, prostate-specific acid phosphatase, prostatic carcinoma-associated complex, p53,

albumin, estrogen receptor, progesterone receptor, cholecystokinin, insulin, luteinizing hormone and salivary amylase.

29. The primers of claim 27 wherein said sequences are within about 70 to about 250 nucleotides of each other.

30. A pair of primers for PCR amplification of nucleic acids specific for prostate specific antigen wherein a sequence of one primer corresponds to an exonic sequence of the prostate specific antigen gene and a sequence of the other primer corresponds to a different exonic sequence of the prostate specific antigen gene and said sequences are within about 250 nucleotides of each other along an expressed region of said gene.

31. The primers of claim 30 wherein said sequences are within about 70 to about 250 nucleotides of each other.

32. The primers of claim 30 wherein said sequences are within about 50 to about 150 nucleotides of each other.

33. The primers of claim 30 wherein the sequence of one primer is selected from the group consisting of:

5'-CGAGAAGCATTCCCAACCCTGGC-3' (SEQ ID NO 1);
5'-GTGCTTGTGGCCTCTCGTGGCAG-3' (SEQ ID NO 5); and
5'-CTCACAGCTGCCCACTGCATCAG-3' (SEQ ID NO 7); and the sequence of said other primer is selected from the group consisting of;
5'-GGGTGAACTTGCGCACACACGTC-3' (SEQ ID NO 2);
5'-CCTGGCCTGTGTCTTCAGGATG-3' (SEQ ID NO 3);
5'-GAGGTCGTGGCTGGAGTCATCAC-3' (SEQ ID NO 4);
5'-GGAGGCTCATATCGTAGAGCGGG-3' (SEQ ID NO 6); and
5'-GTCATCACCTGGCCTGAGGAATC-3' (SEQ ID NO 8).

34. A diagnostic kit for the detection of metastatic prostate carcinoma comprising a pair of primers for PCR amplification of nucleic acids specific for prostate specific antigen wherein a sequence of one primer corresponds to an exonic sequence of the prostate specific antigen gene and a sequence of the other

primer corresponds to a different exonic sequence of the prostate specific antigen gene and said sequences are within about 250 nucleotides of each other along an expressed region of said gene.

35. The kit of claim 34 wherein the sequence of one primer is selected from the group consisting of;

5'-CGAGAAGCATTCCCAACCCTGGC-3' (SEQ ID NO 1);
5'-GTGCTTGTGGCCTCTCGTGGCAG-3' (SEQ ID NO 5); and
5'-CTCACAGCTGCCCACTGCATCAG-3' (SEQ ID NO 7); and the sequence of said other primer is selected from the group consisting of;

5'-GGGTGAACTTGCGCACACACGTC-3' (SEQ ID NO 2);
5'-CCTGGCCTGTGTCTTCAGGATG-3' (SEQ ID NO 3);
5'-GAGGTCGTGGCTGGAGTCATCAC-3' (SEQ ID NO 4);
5'-GGAGGCTCATATCGTAGAGCGGG-3' (SEQ ID NO 6); and
5'-GTCATCACCTGGCCTGAGGAATC-3' (SEQ ID NO 8).

36. The kit of claim 34 further comprising a thermostable DNA polymerase and reagents for PCR amplification reactions.

```
     TTTCCCTT CTCCCAGTCCAA GACCCCAAATCA CCACAAAGGACC CAATCCCCAGAC TCAAGATATGGT CTGGGCGCTGTC TTGTGTCTCCTA   96
                                                                       M V   W A L
             possible splice donor sites
     CCCTGATCCCTG GGTTCAACTCTG CTCCCAGAGCAT GAAGCCTCTCCA CCAGCACCAGCC ACCAACCTGCAA ACCTAGGGAAGA TTGACAGAATTC   192

     CCAGCCTT.CCC AGCTCCCCCTGC CCATGTCCCAGG ACTCCCAGCCTT GGTTCTCTGCCC CCGTGTCTTTTC AAACCCACATCC TAAATCCATCTC   288
                                                    splice acceptor                              mature protein
289  CTATCCGAGTCC CCCAGTTCCTCC TGTCAACCCTGA TTCCCCTGATCT AGCACCCCCTCT GCAGGTGCTGCA CCCCTCATCCTG TCTCGGATTGTG   384
                                                                       A A   P L I L   S R I V
385  GGAGGCTGGGAG TGCGAGAAGCAT TCCCAACCCTGG CAGGTGCTTGTA GCCTCTCGTGGC AGGGCAGTCTGC GGCGGTGTTCTG GTGCACCCCCAG   480
     G G W E      C E K H      S Q P W      Q V L V      A S R G      R A V C      G G V L      V H P Q
481  TGGGTCCTCACA GCTACCCACTGC ATCAGGAACAAA AGCGTGATCTTG CTGGGTCGGCAC AGCCTGTTTCAT CCTGAAGACACA GGCCAGGTATTT   576
     W V L T      A T H C      I R N K      S V I L      L G R H      S L F H      P E D T      G Q V F
                    N
577  CAGGTCAGCCAC AGCTTCCCACAC CCGCTCTACGAT ATGAGCCTCCTG AAGAATCGATTC CTCAGGCCAGGT GATGACTCCAGC CACGACCTCATG   672
     Q V S H      S F P H      P L Y D      M S L L      K N R F      L R P G      D D S S      H D L M
           R
673  CTGCTCCGCCTG TCAGAGCCTGCC GAGCTCACGGAT GCTATGAAGGTC ATGGACCTGCCC ACCCAGGAGCCA GCACTGGGGACC ACCTGCTACGCC   768
     L L R L      S E P A      E L T D      A M K V      M D L P      T Q E P      A L G T      T C Y A
                                             V
769  TCAGGCTGGGGC AGCATTGAACCA GAGGAGTTCTTG ACCCCAAAGAAA CTTCAGTGTGTG GACCTCCATGTT ATTTCCAATGAC GTGTGTGCGCAA   864
     S G W G      S I E P      E E F L      T P K K      L Q C V      D L H V      I S N D      V C A Q
                                  H L L Y D Q H
865  GTTCACCCTCAG AAGGTGACCAAG TTCATGCTGTGT GCTGGACGCTGG ACAGGGGGCAAA AGCACCTGCTCG GGTGATTCTGGG GGCCCACTTGTC   960
     V H P Q      K V T K      F M L C      A G R W      T G G K      S T C S      G D S G      G P L V
961  TGTAATGGTGTG CTTCAAGGTATC ACGTCATGGGGC AGTGAACCATGT GCCCTGCCCGAA AGGCCTTCCCTG TACACCAAGGTG GTGCATTACCGG   1056
     C N G V      L Q G I      T S W G      S E P C      A L P E      R P S L      Y T K V      V H Y R
1057 AAGTGGATCAAG GACACCATCGTG GCCAACCCCTGA GCACCCCTATCA ACTCCCTATTGT AGTAAACTTGGA ACCTTGGAAATG ACCAGGCCAAGA   1152
     K W I K      D T I V      A N P *
1153 CTCAAGCCTCCC CAGTTCTACTGA CCTTTGTCCTTA GGTGTGAGGTCC AGGGTTGCTAGG AAAAGAAATCAG CAGACACAGGTG TAGACCAGAGTG   1248
1249 TTTCTTAAATGG TGTAATTTTGTC CTCTCTGTGTCC TGGGGAATACTG GCCATGCCTGGA GACATATCACTC AATTTCTCTGAG GACACAGATAGG   1344
1345 ATGGGGTGTCTG TGTTATTTGTGG GGTACAGAGATG AAAGAGGGGTGG GATCCACACTGA GAGAGTGGAGAG TGACATGTGCTG GACACTGTCCAT   1440
1441 GAAGCACTGAGC AGAAGCTGGAGG CACAACGCACCA GACACTCACAGC AAGGATGGAGCT GAAAACATAACC CACTCTGTCCTG GAGGCACTGGGA   1536
1537 AGCCTAGAGAAG GCTGTGAACCAA GGAGGGAGGGTC TTCCTTTGGCAT GGGATGGGGATG AAGTAAGGAGAG GGACTGACCCCC TGGAAGCTGATT   1632
1633 CACTATGGGGGG AGGTGTATTGAA GTCCTCCAGACA ACCCTCAGATTT GATGATTTCCTA GTAGAACTCACA GAAATAAAGACC TGTTATACTGTG   1728
1729 AApolyA   1735                                                   polyadenylation signal
```

(SEQ ID NO 13)

FIGURE 1

```
   1  GGTGTCTTAG GCACACTGGT CTTGGAGTGC AAAGGATCTA GGCACGTGAG GCTTTGTATG AAGAATCGGG GATCGTACCC ACCCCCTGTT TCTGTTTCAT
 101  CCTGGGCATG TCTCCTCTGC CTTTGTCCCC TAGATGAAGT CTCCATGAGC TACAAGGCCC TGGTCCATCC AGGGTGATCT AGTAATTGC AGAACAGCAAG
                                                      steroid receptor binding consensus sequence──────┘
 201  TGCTAGCTCT CCCTCCCCTT CCACAGCTCT GGGTGTGGGA GGGCGTTGTC CAGCCTCCAG CAGCATGGGG AGGGCCTTGG TCAGCCTCTG GGTGCCAGCA
                               putative TATA box
 301  GGGCAGGGGC GGAGTCCTGG GGAATGAAGG TTTTATAGGG CTCCTGGGGG AGGCTCCCCA GCCCCAACCT TACCACCTGC ACCCGGAGAG CTGTGTCACC
 401  ATGTGGGTCCCG GTTGTCTTCCTC ACCCTGTCCGTG ACGTGGATTG GTGA CAGGGGCCAT GGTTGGGGGG ATGCAGGAGA GGGAGCCAGC CCTGACTGTC
      M  W  V  P   V  V  F  L   T  L  S  V   T  W  I  G  └──splice donor |
 501  AAGCTGAGGC TCTTTCCCCC CCAACCCAGC ACCCCAGCCC AGACAGGGAG CTGGGCTCTT TTCTGTCTCT CCCAGCCCCA CTTCAAGCCC ATACCCCCAG
 601  TCCCCTCCAT ATTGCAACAG TCCTCACTCC CACACCAGGT CCCCCCTCCC TCCCACTTAC CCCAGAACTT TCTTCCCATT TGCCCAGCCA GCTCCCTGCT
 701  CCCAGCTGCT TTACTAAAGG GGAAGTTCCT GGGCATCTCC GTGTTTCTCT TTGTGGGGCT CAAAACCTCC AAGGACCTCT CTCAATGCCA TTGGTTCCTT
 801  GGACCGTATC ACTGGTCCAT CTCCTGAGCC CCTCAATCCT ATCACAGTCT ACTGACTTTT CCCATTCAGC TGTGAGTGTC CAACCCTATC CCAGAGACCT
 901  TGATGCTTGG CCTCCCAATC TTGCCCTAGG ATACCCAGAT GCCAACCAGA CACCTCCTTC TTTCCTAGCC AGGCTATCTG GCCTGAGACA ACAAATGGGT
1001  CCCTCAGTCT GGCAATGGGA CTCTGAGAAC TCCTCATTCC CTGACTCTTA GCCCCAGACT CTTCATTCAG TGGCCCACAT TTTCCTTAGG AAAAACATGA
1101  GCATCCCCAG CCACAACTGC CAGCTCTCTG AGTCCCCAAA TCTGCATCCT TTTCAAAACC TAAAAACAAA AAGAAAAACA AATAAAACAA AACCAACTCA
1201  GACCAGAACT GTTTTCTCAA CCTGGGACTT CCTAAACTTT CCAAAACCTT CCTCTTCCAG CAACTGAACC TCGCCATAAG GCACTTATCC CTGGTTCCTA
1301  GCACCCCTTA TCCCCTCAGA ATCCACAACT TGTACCAAGT TTCCCTTCTC CCAGTCCAAG ACCCCAAATC ACCACAAAGG ACCCAATCCC CAGACTCAAG
1401  ATATGGTCTG GGCGCTGTCT TGTGTCTCCT ACCCTGATCC CTGGGTTCAA CTCTGCTCCC AGAGCATGAA GCCTCTCCAC CAGCACCAGC CACCAACCTG
1501  CAAACCTAGG GAACATTGAC AGAATTCCCA GCCTTTCCCA GCTCCCCCTG CCCATGTCCC AGGACTCCCA GCCTTGGTTC TCTGCCCCCG TGTCTTTTCA
1601  AACCCACATC CTAAA;CCAT CTCCTATCCG AGTCCCCCAG TTCCCCCTGT CAACCCTGAT TCCCCTGATC TAGCACCCCC TCTGCAG GCGCTGGGCCCCTC
                                                                           splice acceptor──┘   A  A  P  L
                   ┌────mature protein
1702  ATCCTGTCTCGG ATTGTGGGAGGCTGG  1728
      I  L  S  R   I  V  G  G  W
```

(SEQ I D No 14)

FIGURE 2

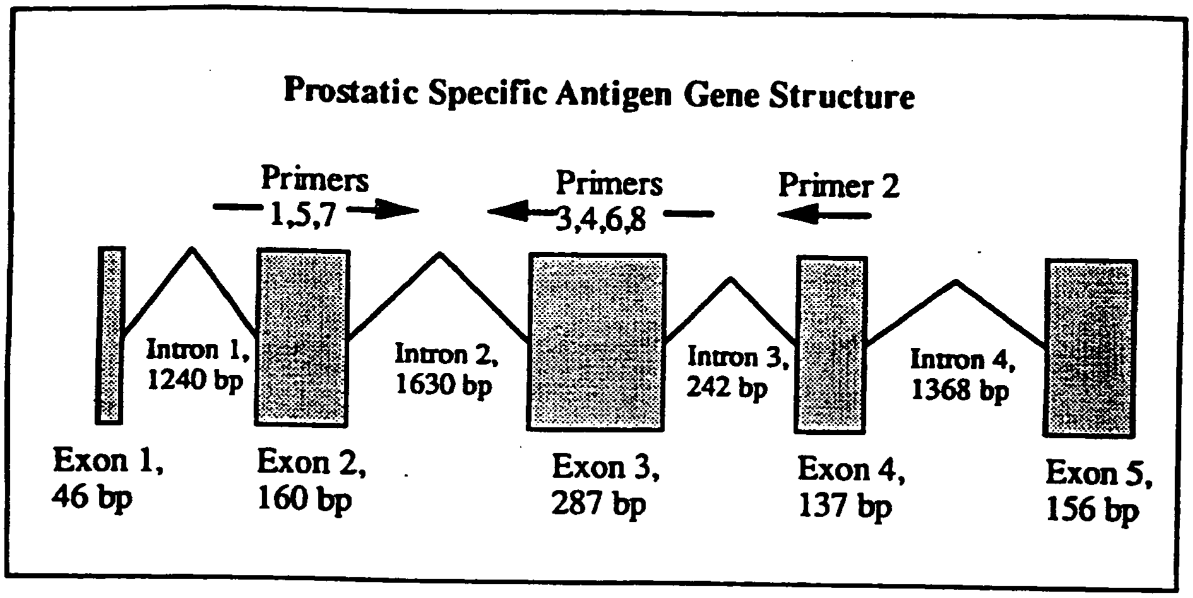
Prostatic Specific Antigen Gene Structure
Primers
1,5,7
Primers
3,4,6,8
Primer 2
Intron 1,
1240 bp
Intron 2,
1630 bp
Intron 3,
242 bp
Intron 4,
1368 bp
Exon 1,
46 bp
Exon 2,
160 bp
Exon 3,
287 bp
Exon 4,
137 bp
Exon 5,
156 bp

FIGURE 3

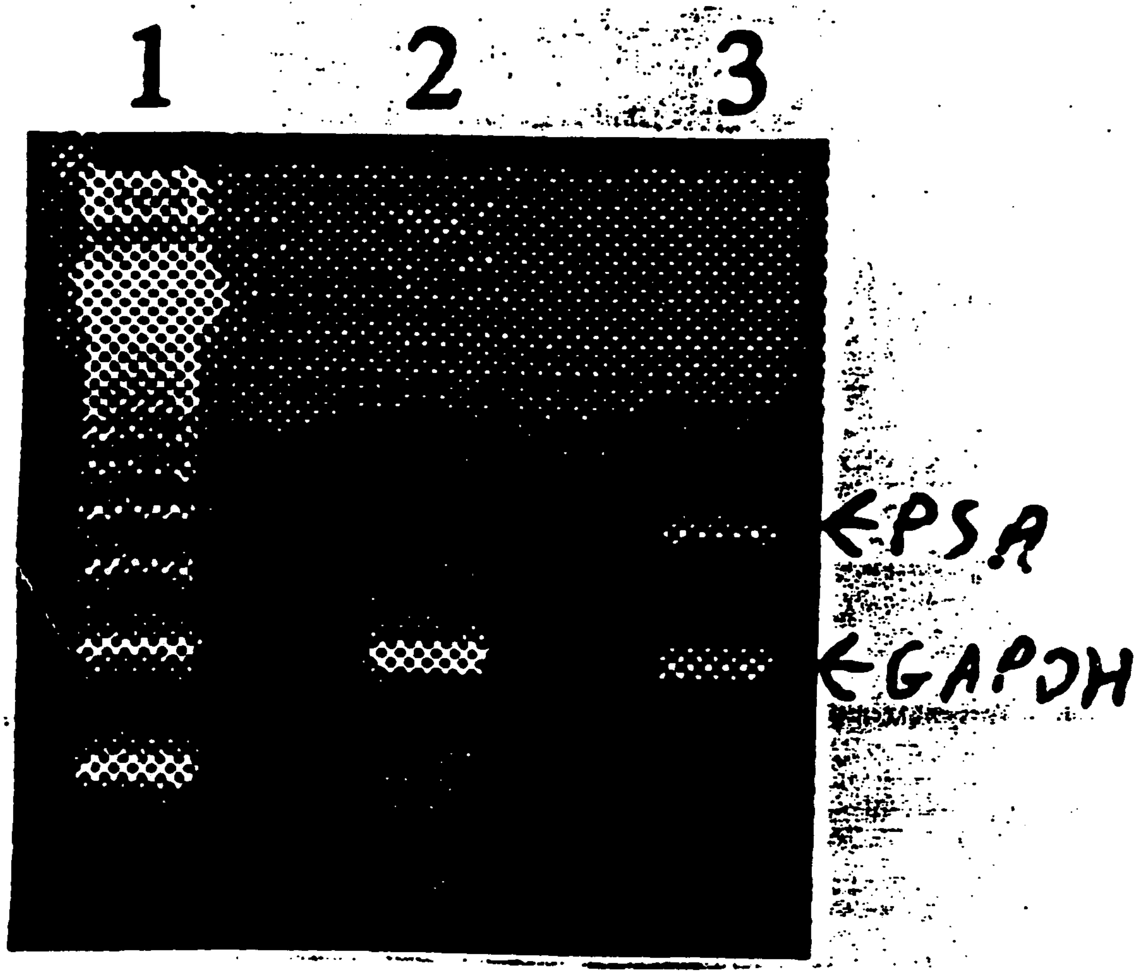

FIGURE 4

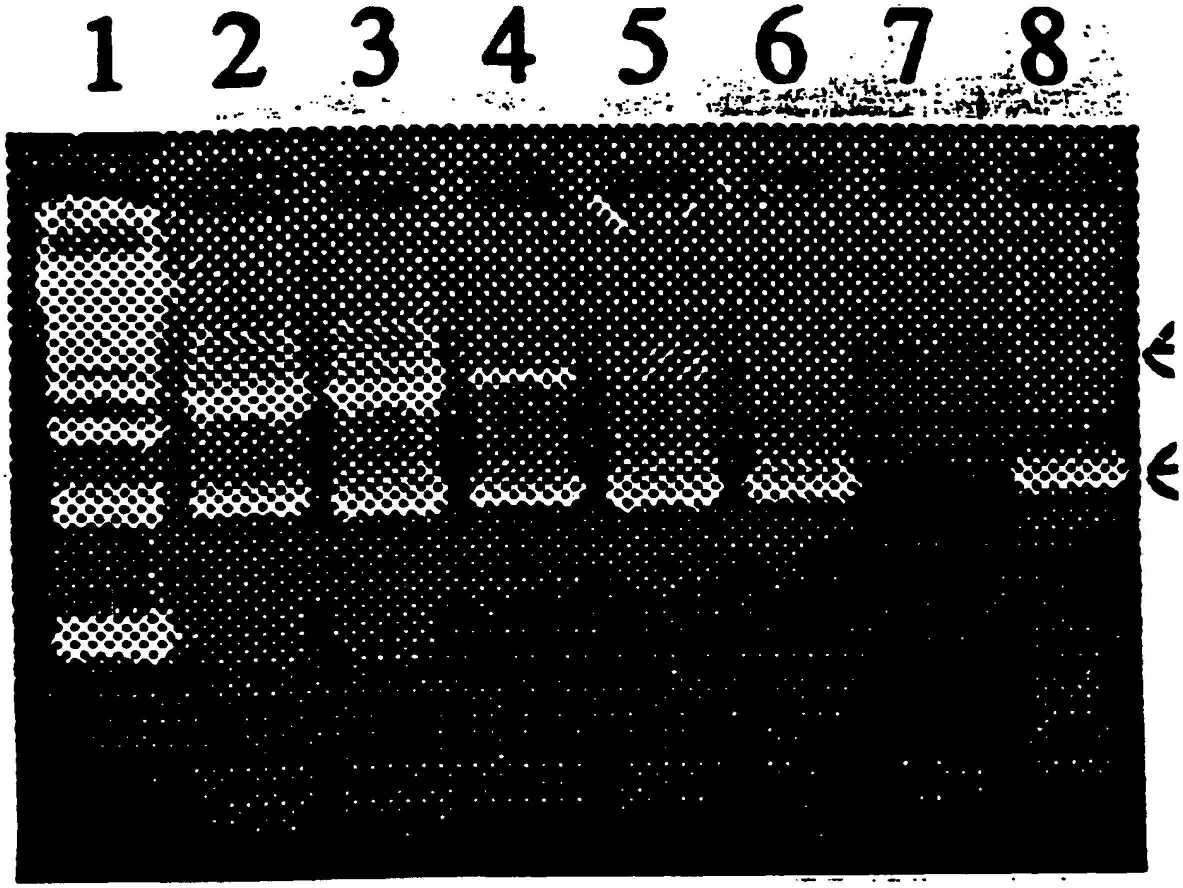

FIGURE 5

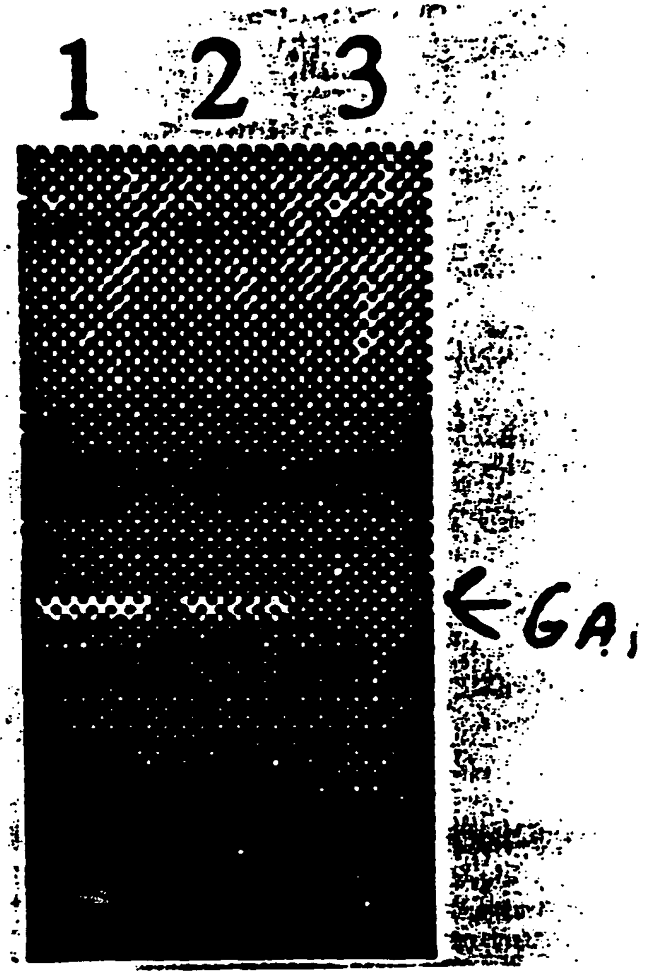

FIGURE 6

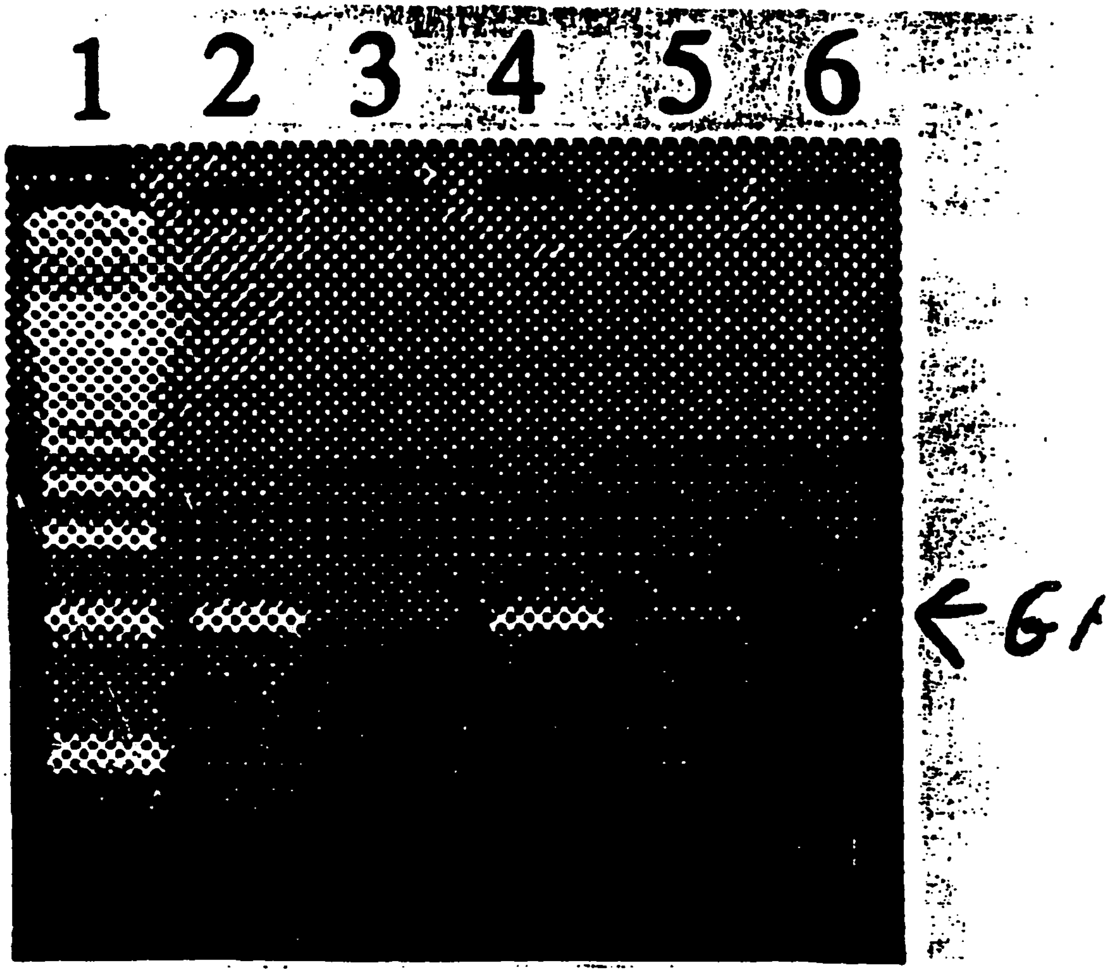

FIGURE 7

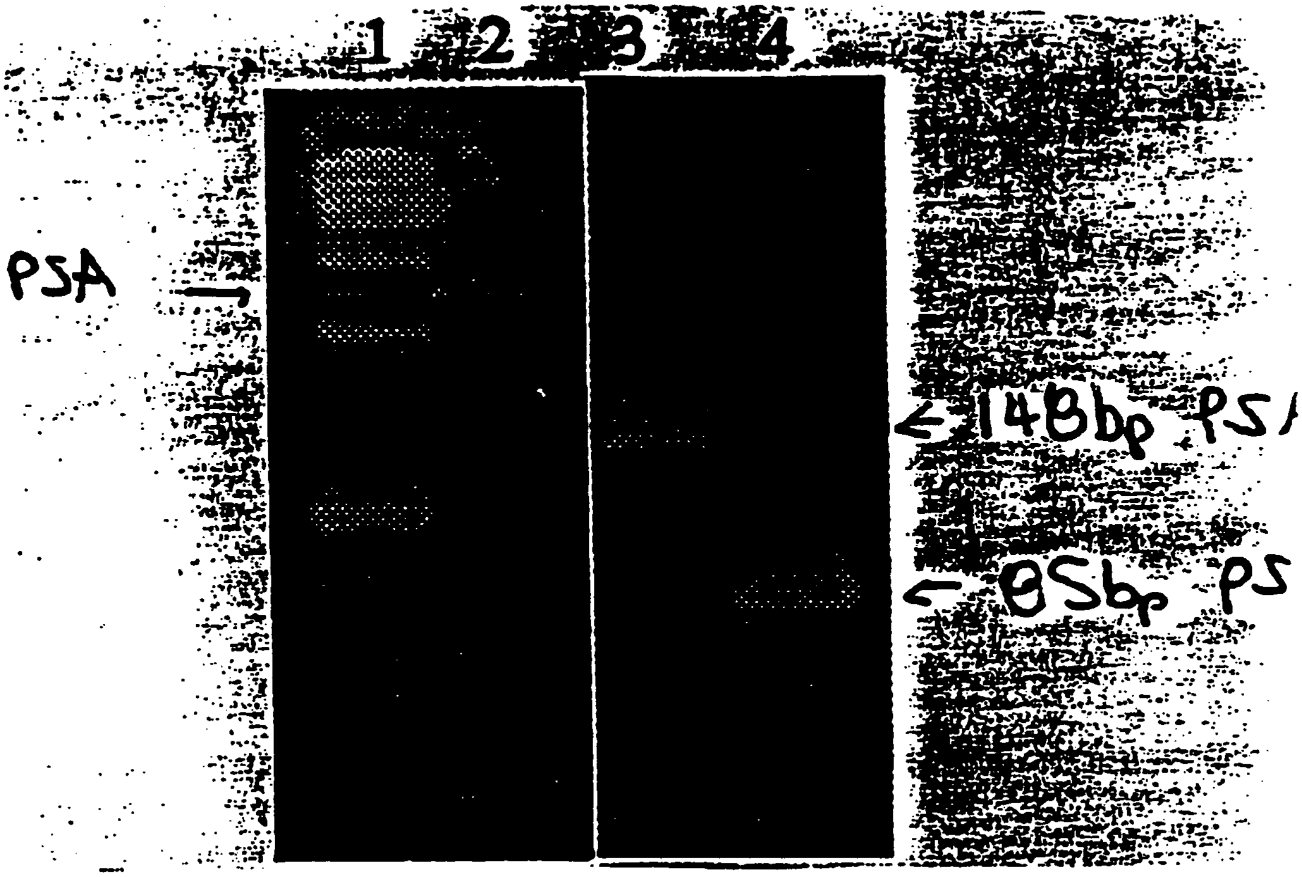

FIGURE 8

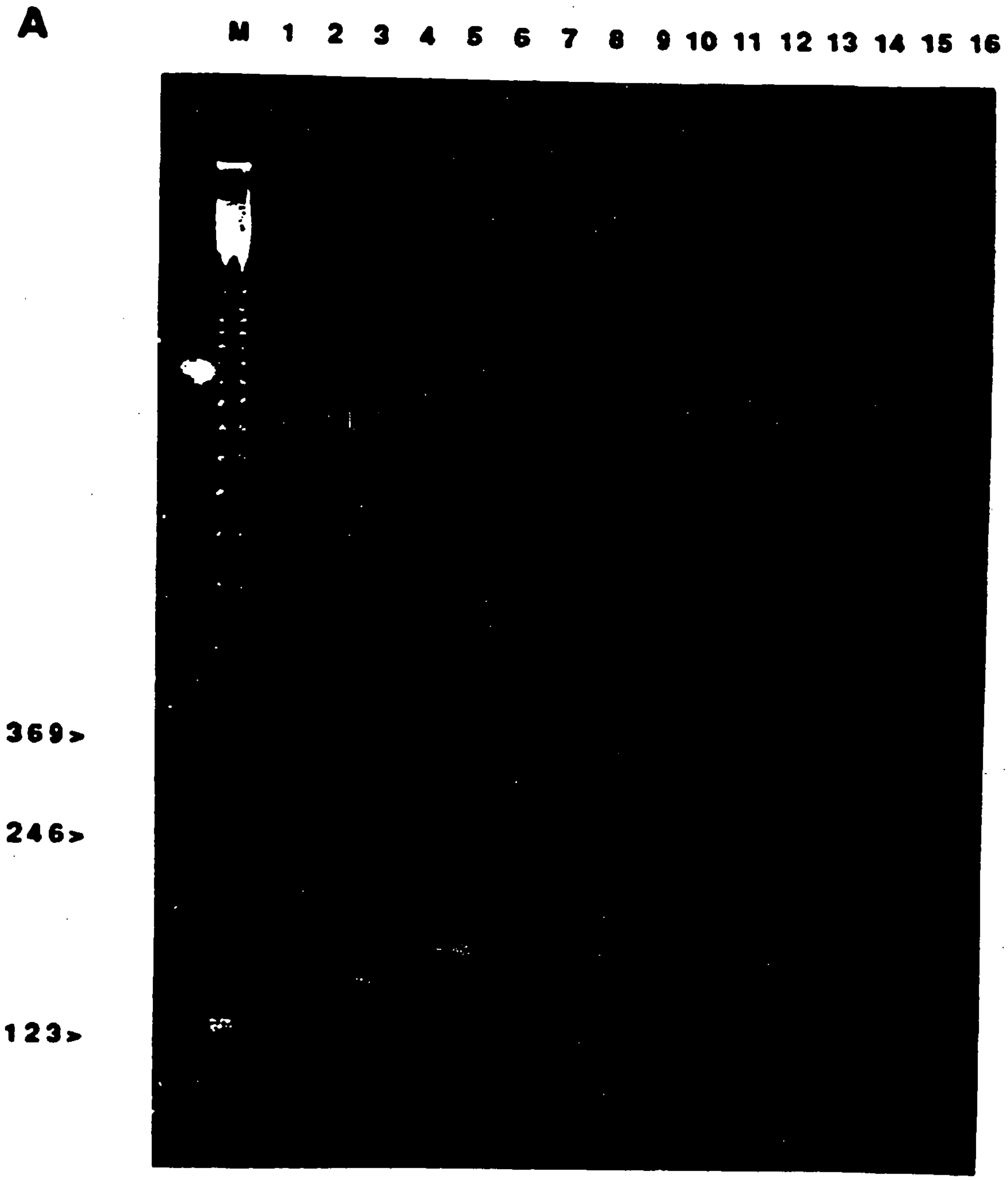

FIGURE 9

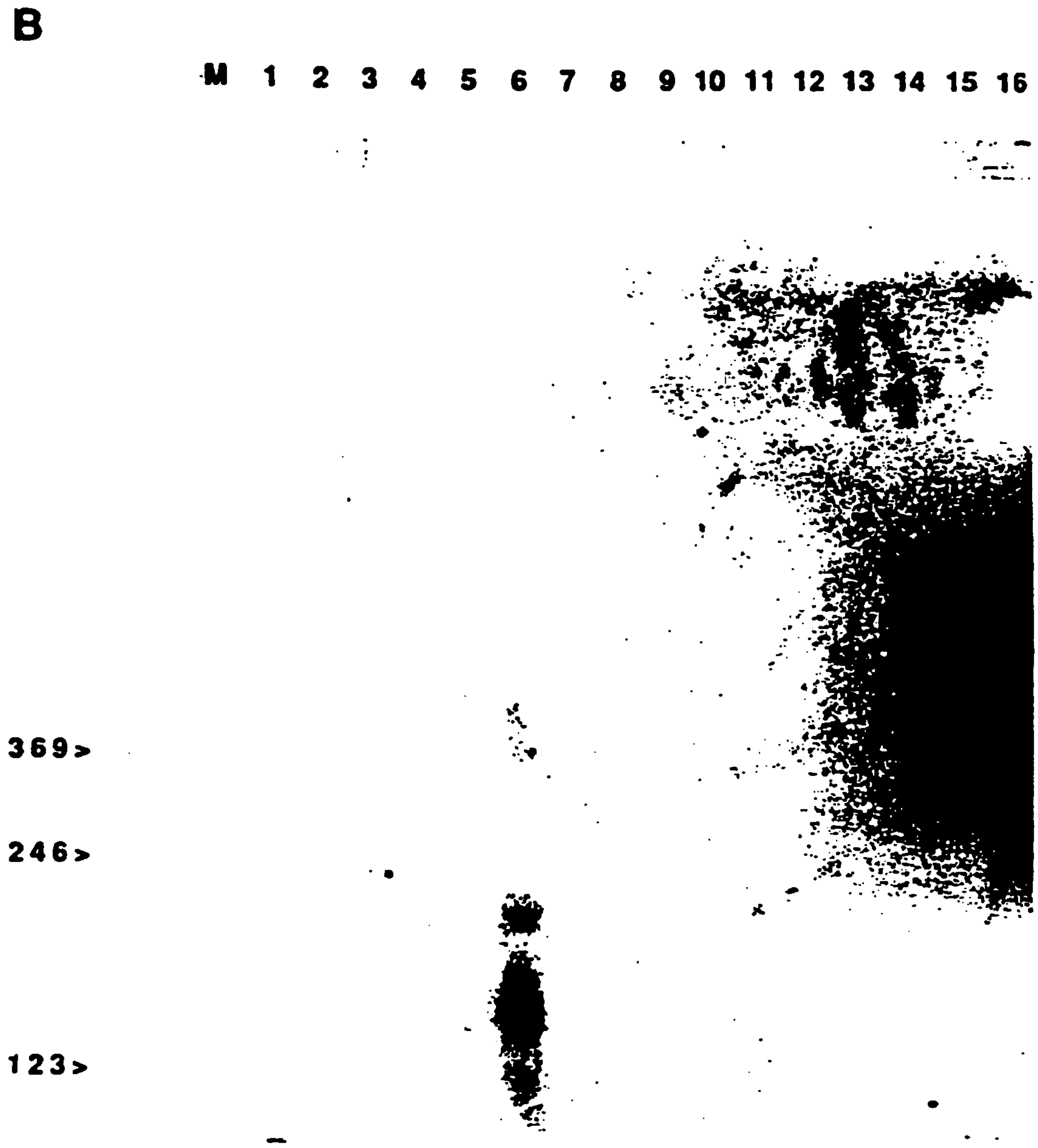

FIGURE 10

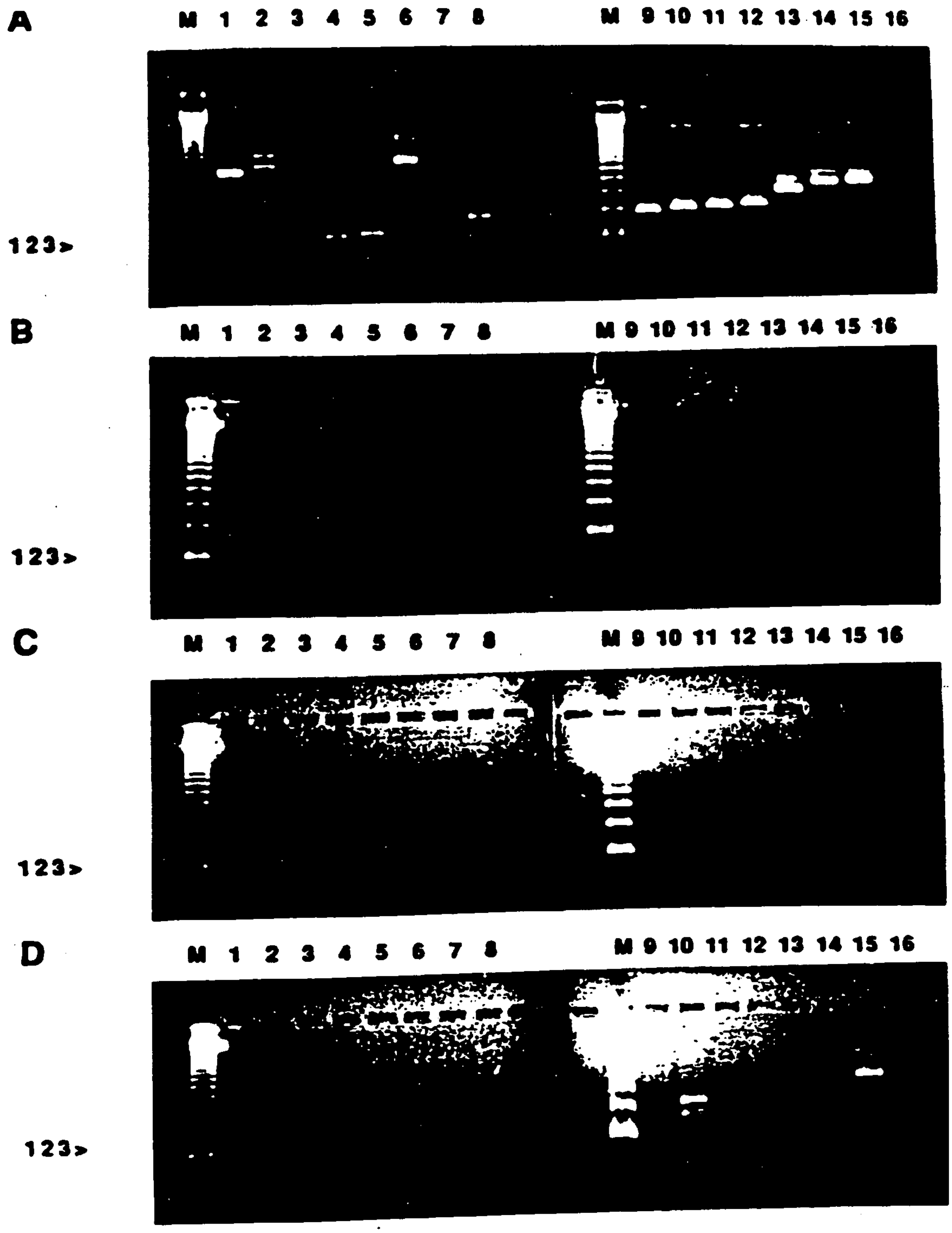
A
M 1 2 3 4 5 6 7 8
M 9 10 11 12 13 14 15 16
123>
B
M 1 2 3 4 5 6 7 8
M 9 10 11 12 13 14 15 16
123>
C
M 1 2 3 4 5 6 7 8
M 9 10 11 12 13 14 15 16
123>
D
M 1 2 3 4 5 6 7 8
M 9 10 11 12 13 14 15 16
123>

FIGURE 11

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